Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Biofilm formation by *Azospirillum brasilense* – Microbial socialization in the rhizosphere

Francisco Diogo de Almeida Cerqueira

Dissertação

MESTRADO EM MICROBIOLOGIA APLICADA

2014

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Biofilm formation by *Azospirillum brasilense* – Microbial socialization in the rhizosphere

Francisco Diogo de Almeida Cerqueira

Dissertação

Mestrado em Microbiologia Aplicada

Dissertação orientada pela Doutora Cristina Cruz
(FCUL, DBV, CBA) e pelo Doutor Luís Carvalho
(FCUL, CBA)

2014

Biofilm formation by *Azospirillum brasilense* – Microbial socialization in the rhizosphere

Francisco Diogo de Almeida Cerqueira

2014

This thesis was fully performed at the Center for Environmental Biology (CBA), Faculty of Sciences of the University of Lisbon, under the direct supervision of Doctor Luís Carvalho.

Doctor Cristina Cruz was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.

Acknowledgements

This work was carried out in fulfillment of the Master in Applied Microbiology at the Faculty of Sciences of the University of Lisbon, being performed at Center for Environmental Biology (CBA), Faculty of Sciences of the University of Lisbon. I would like to acknowledge, Dr. Cristina Cruz, for providing the necessary support and means to perform this work.

I would like to thank my parents and my friends for their immeasurable support which culminated in the opportunity of accomplishing this dissertation.

I would like to acknowledge the group of Biotic-Abiotic Interactions, for their precious guidance in the laboratory since the beginning of this work.

Last but definitely not least, I am incredibly grateful to my co-supervisor Luís Carvalho for his patience and guidance.

Abstract

Azospirillum brasilense is a remarkable Plant-Growth Promoting Rhizobacteria (PGPR), with great potential as agricultural inoculant for several crops. Several isolates of this species have been produced as inoculant by some companies and applied in the agriculture as a biofertilizer. Therefore, there is a great interest in gathering more scientific knowledge that improves *A. brasilense* efficacy as a PGPR.

To enhance the beneficial effects, from phytohormone synthesis to nitrogen fixation, it is essential that *A. brasilense* colonize plant roots and survive in the soil. For such, a vast array of traits is required, including biofilm formation and chemotactic response. The rhizosphere is a hotspot of biodiversity and a place of intensive networking; root exudates are the major plant tool to modulate the reciprocal interactions between the plant and the rhizosphere. In this work I focus on the influence of root symbiotic Arbuscular Mycorrhizal Fungi (AMF) and rhizospheric bacteria on *A. brasilense* biofilm formation with the following objectives: Firstly, to test if root exudates from maize plants colonized or not with different AMF species generate chemotactic response and stimulate biofilm formation of *A. brasilense.* Secondly, to test the interspecific influence of several soil bacterial strains in *A. brasilense* biofilm formation. Moreover, to assess the effect of *Pseudomonas putida* X236 and *Bradyrhizobium japonicum* by growing *A. brasilense* with supernatants, in co-inoculation, and with root exudates from maize plants inoculated with each one of those strains and with *A. brasilense* itself. Finally, inquire Phytohormone production, namely indol-3-acetic-acid (IAA), of *A. brasilense* in biofilm to assess a potential benefit to plants.

Results showed that some mycorrhizal fungal species interact with *A. brasilense* via root exudates acting as chemoatractants. The soil borne bacteria *P. putida* X236 promoted biofilm formation of *A. brasilense*, indicating potential to create a co-inoculum with *A. brasilense.* Root exudates of maize plants inoculated with *A. brasilense* provoke a raise in its biofilm. IAA levels in biofilm cells were generally higher than in planktonic cells. This study highlights the importance of interspecific relations and of microbial consortia inoculums to maximize functions of the PGPR *A. brasilense*.

Resumo

Azospirillum brasilense é uma Rizobactéria Promotora do Crescimento de Plantas, com grande potencial para uso como inoculante agrícola em diversas culturas de plantas. Foram já desenvolvidos inóculos com aplicação na agricultura como biofertilizante. Portanto, há um grande interesse em obter conhecimento que possa melhorar a eficácia de *A. brasilense*.

Para aumentar os efeitos benéficos potenciais do *A. brasilense* como a síntese de fito-hormonas e a fixação de azoto, é essencial que *A. brasilense* consiga colonizar eficazmente as raízes, possa perdurar no solo e tenha as condições necessárias para manifestar o fenótipo adequado o que inclui a formação de biofilme e a resposta quimiotáctica a exsudados radiculares. A rizosfera é um "hotspot" de biodiversidade e palco de inúmeras relações entre organismos, sendo os exsudados radiculares o meio pelo qual a raiz influência a estrutura e função do microbioma rizosférico. Neste contexto o meu trabalho de mestrado foi centrado no estudo da influência de fungos micorrízicos arbusculares (AMF) e bactérias rizosféricas na formação de biofilme em *A. brasilense* e teve como objectivos: Em primeiro lugar, testar exsudados radiculares de plantas de milho colonizadas ou não por diferentes espécies de AMF, e analisar a resposta quimiotática e a formação de biofilme por *A. brasilense*. Em segundo lugar, avaliar o efeito de interacções de várias estirpes de bactérias presentes no solo na formação de biofilme de *A. brasilense*. Adicionalmente, estudar-se mais aprofundadamente o efeito de *Pseudomonas putida* X236 e *Bradyrhizobium japonicum*, crescendo *A. brasilense* com sobrenadantes, em co-inoculação e com exsudados radiculares recolhidos de plantas de milho inoculadas com cada uma dessas estirpes e do próprio *A. brasilense*. Por último, avaliar a síntese de fito-hormonas, nomeadamente de indol-3-acético (IAA), por parte de *A. brasilense* em biofilme para averiguar a influência de um potencial efeito benéfico nas plantas.

Os resultados deste estudo demonstraram que certas espécies de AMF interagem com *A. brasilense* via exsudados, actuando como quimioatratores. A bactéria *P. putida* X236 estimulou a formação de biofilme de *A. brasilense*, indicando potencial para formar um co-inóculo com *A. brasilense*. Exsudados de plantas de milho inoculadas com *A. brasilense* promoveram um aumento do seu biofilme. Níveis de IAA sintetizado em biofilmes foram geralmente superiores aos das células planctónicas, indicando a importância da colonização radicular de *A. brasilense* pela forma de biofilme. Este trabalho realça a importância das relações interespecíficas e o potencial do uso de consórcios microbianos para maximizar funções de *A. brasilense* como PGPR.

Index

I - Introduction

I.1 - Rhizosphere

The rhizosphere is the narrow zone of soil that surrounds and is influenced by plant roots. The rhizosphere is a complex environment with an enormous microbial biodiversity, where it is possible to find countless and highly dynamic interactions, either positive or negative, between roots and soil borne microorganisms. These organisms can have profound effects on the growth, nutrition and plant health, and they can also affect the composition and biomass of plant communities in natural ecosystems (1).

I.2 - Biofilms

The vast majority of bacteria in their natural habitat tend to persist as a community named biofilm attached to a surface in a self-produced extracellular matrix, and not as free living form (2). They usually appear between interfaces, like air-liquid or liquid solid. A biofilm can be composed by just one species or, more commonly, can be formed by several bacterial species, forming a multicellular complex that requires coordination and has a complex behavior (3).

I.2.1 - Quorum sensing

Biofilm formation is a controlled process, in which bacteria communicate cell to cell by Quorum sensing. It involves production, detection and response of the signaling molecules named autoinducers (AI) (4). In most gram-negative bacteria the AIs fall in the category of Acylated homoserine lactones (AHLs), and in gram-positive AIs are peptides (5). These molecules allow bacteria to sense population density, by assessing the accumulation of AIs. AIs diffuse away, and, therefore, are present at concentrations below the threshold required for detection. At high cell density, the cumulative production of AIs leads to a local higher concentration, enabling detection and response (6).

Quorum sensing does not only have a major role in the early stages of biofilms, but it also regulates the whole process and even cell detachment when the cell density is too high after the maturation, and they need to disperse for other niches colonization (4).

I.2.2 - Development

Biofilms form in response to environmental stimuli and they are not a static entity, being a part of biological cycle composed by several stages: initiation, maturation, maintenance and dispersal. During initiation planktonic bacteria adhere to a surface and this initial phase is dependent on motility, cell to cell interactions and adhesion factors, for attachment. Afterwards biofilms maturate, with high synthesis of exopolysaccharides (EPS), and increased resistance to external stress factors. At last bacteria disperse and return to a planktonic growth mode, due to nutrient starvation, high cell density, and other factors that might act as a signal, therefore bacteria may colonize new niches (Fig. 1) (3).

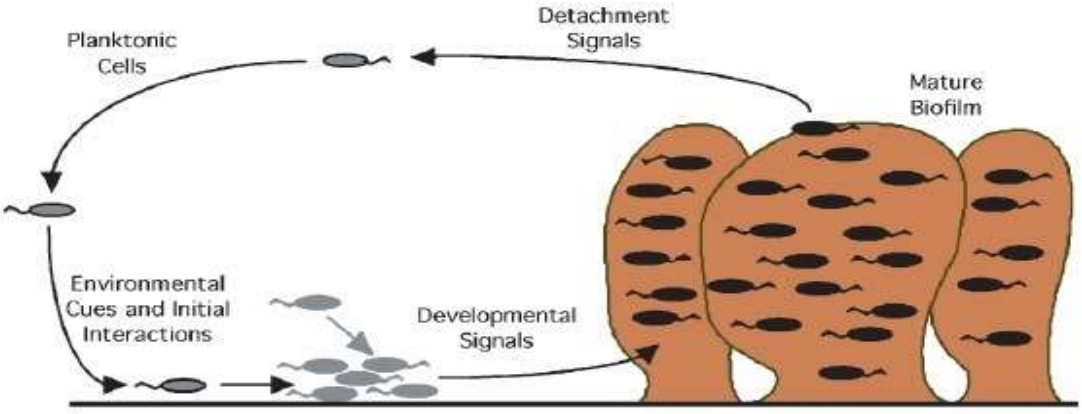


Fig. 1: Scheme of model of biofilm development. Planktonic cells receive environmental stimuli and initiate cell-to-cell communication resulting in the formation of microcolonies, and then maturation begins with synthesis extracellular matrix followed by maintenance of the biofilm while environmental conditions allow it. Cells in the biofilm can return to a mobile state to complete the cycle of biofilm development. Adapted from (3).

I.2.3 - Ecological advantages

Biofilms give several ecological advantages for the bacteria, being the most important protection, cooperation and gene transfer.

Protection is given by a matrix composed mostly by EPS, nucleic acids and proteins (7). Acts as a physical barrier preventing certain antimicrobial agents going into the biofilm by acting as an ion exchanger, thereby restricting diffusion of compounds from the surrounding area into the biofilm (8). This propriety is dependent on the characteristics of both the agent and the EPS matrix. The effect appears to be most pronounced with antibiotics that are hydrophilic and positively charged, such as the aminoglycosides (9, 10). Studies have shown that EPS has also

been reported to sequester metals, cations, and toxins (11, 12), and to protect from physical stress from the environment like pH, UV radiation dissecation (2). Bacteria in multispecies biofilms have some sort of metabolic cooperation, as some bacterial exchange of nutrients, where some use others metabolites, thus removing them, which can be useful since they could be toxic (12). Biofilms are also an excellent niche for gene transfer between bacteria, by conjugation for plasmid transfer. It occurs at a higher rate between cells in biofilms than between planktonic cells (14, 15).

I.2.4 - Biofilms in Plants

Plants change their surrounding environment including the soil, creating niches for microorganisms such as bacteria to prosper. Roots exudate macronutrients into the soil, making them available for bacteria, and they colonize and aggregate as biofilms, although biofilms may be present in the whole plant surface, from the roots to the leaves (Fig. 2) (16).

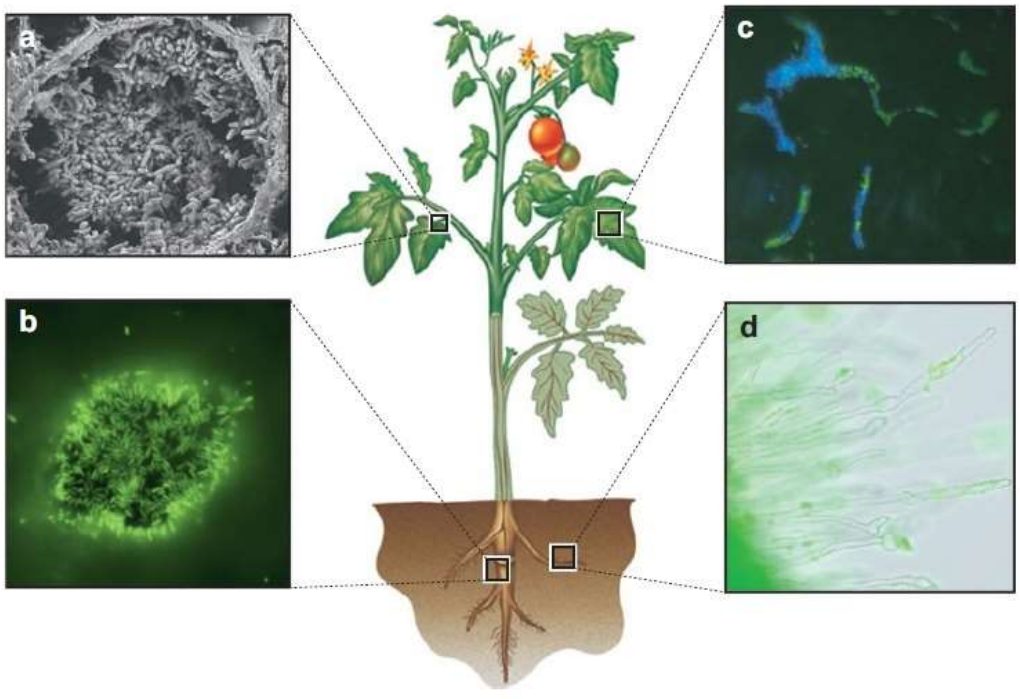


Fig. 2: Scheme representing Tomato plant and the wide distribution of biofilms over plant surface. Subtitles: (*a*) transmission electron micrograph of sweet corn leaf xylem vessel infected by *Pantoea stewartii;* (b) fluorescence microscopy of *Agrobacterium tumefaciens* microcolony*;* (c) fluorescence microscopy of bean leaf with *Pseudomonas syringae* and *Pantoea agglomerans* and (d); fluorescence and light microscopy of alfalfa root hairs with *Sinorhizobium melioti.* Adapted from (16).

I.2.5 - *Azospirillum brasilense* characteristics and biofilm

One of the most studied rhizobacteria is *Azospirillum brasilense* due to its role in plant root system. *A. brasilense* is a plant growth-promoting rhizobacteria (PGPR) (17). It is a PGPR included in the class of alpha proteobacteria, which promotes growth and yield of agronomic and ecological important plant species (18, 19, 20). It can be found in a wide range of habitats associated with roots of various types of plant species. *A. brasilense* was found to possess no obligate specificity in respect to the host plant (21, 22).

A. brasilense is well known for its capability of plant growth promotion by phytohormone synthesis, particularly the auxin indole-3-acetic acid (IAA) (23), nitrogen fixation, converting atmospheric nitrogen into ammonium under microaerobic conditions, through the action of the nitrogenase complex (24). Some studies showed that it is capable of nitric oxide production, inducing lateral root formation in tomato seedlings (25). There are not much studies about its biocontrol competencies, nonetheless it has been shown that *A. brasilense* contributes for crown gall disease, bacterial leaf blight of mulberry, and bacterial leaf and vascular diseases of tomato biocontrol (26, 27, 28, 29). Furthermore it synthetizes phenylacetic acid (PAA), which has antibacterial and antifungic activity (30).

In addition to all these competencies, *A. brasilense* is capable to colonize and form biofilm in plant root surface. Root exudates and oxygen concentration create taxis towards the roots. The carbon compound chemotaxis is strain-specific, reflecting the variability of root exudation by different host plants (31, 32).

In most gram negative bacteria AHLs are the molecules responsible for quorum sensing, though most *Azospirillum* strains do not synthetize AHLs (33, 34). Aerotaxis is also extremely important for *A. brasilense* to meet the right oxygen concentration that allows respiration and nitrogenase function for nitrogen fixation (35).

Afterwards, in attachment and aggregation, *A. brasilense* uses an arabinose-containing polysaccharide (36) and outer membrane proteins, as major outer membrane protein (MOMP) (37, 38). Tad pili is supposed to be involved in attachment to the root surface and biofilm formation in *A. brasilense* sp245, instead of type IV pili which is used by most bacteria (39).

Several factors may act as stimuli for *A. brasilense* biofilm formation, including signaling molecules synthetized by itself, plants or other microorganisms. Recently it has been shown that 2, 4-diacetylphloroglucinol (DAPG), a secondary metabolite produced by some bacteria of *Pseudomonas* genus, has a positive effect on biofilm formation at intermediate concentrations (40). Other studies shown that NO, a signaling molecule with a wide variety of functions,

influence biofilm formation of several bacteria including *A. brasilense* sp245, possibly acting as early signal in biofilm development (41).

I.3 - Mycorrhizal fungi

Mycorrhizal fungi are symbionts in the roots of the majority of higher plants, enhancing plant nutrition, stress resistance and pathogenic resistance (42). Fungi colonize the host plant's roots, being either intracellularly as in arbuscular mycorrhizal fungi (AMF), or extracellularly as in Ectomycorrhizae fungi (ECM). All AMF belong to the phylum Glomeromycota (43), while ECM belong to the phyla Basidiomycota, Ascomycota and Zygomycota (44). Many agriculturally and horticulturally important crop species are included on the terrestrial plants that form a symbiotic association with AMF (42).

AMF and bacteria naturally co-exist in the rhizosphere. Interactions between AMF and PGPR most likely occur, and can modulate plant health and productivity. Many non-symbiotic rhizosphere bacteria fix atmospheric nitrogen making it available for plants. There are reports of positive interaction between free-living N_2-fixing bacteria and AMF (45), including one study suggesting that AMF species may have stimulating effects on nitrogen fixer *A. brasilense* (46). Although free-living bacteria contribute for nitrogen availability, symbiotic bacteria of plant nodules are responsible for most of nitrogen fixation in plants and studies have revealed that nodulation by indigenous rhizobia is greatly improved by AMF (47).

Studies have shown that some bacterial species respond to the presence of certain AMF suggesting a high degree of specificity between bacteria associated with AMF. One possible explanation for this stimulation of certain bacterial species by specific AMF may be due to those bacteria being activated by species-specific fungal exudates (48, 49). Therefore, AMF can potentially shape soil microbial community composition and function by acting as large suppliers of carbon to the rhizosphere (50).

I.4 - Microbial Socialization

In the rhizosphere there are interactions between plants and those microorganisms and amongst themselves. Bacteria may act as a biocontrol agent, inhibiting growth of pathogenic

microorganisms, or can have a mutualistic or commensal interaction with other bacteria or fungus (16).

Most studies have focused on root-secreted compounds on the selection of root-associated bacteria and the control of their plant-beneficial activities; bacterial secondary metabolites, including phytohormones, on root growth and plant defense; and quorum-sensing pheromones in the regulation of microbe–microbe social relationships (40).

Members of Pseudomonadaceae family are known for their role in the rhizosphere. Various strains of *Pseudomonas putida* and *Pseudomonas fluorescens* produce antifungical metabolites, siderophores and hydrogen cyanide, that inhibit growth of several pathogenic fungi, especially *Fusarium oxysporum* (51, 52, 53). Sometimes bacteria not only inhibit growth of other organism by their secondary metabolites, but by nutrient or niche competition, as it can occur in *P. fluorescens* PCL1751 (51, 54). Other strains of *P. fluorescens* synthetize DAPG, a phenolic compound that can increase gene expression, particularly the genes responsible for cell motility, biofilm formation, poly-β-hydroxybutyrate, and auxin production of *A. brasilense* (40).

Not only pseudomonads act against phytopathogens, gram positive bacteria as *Bacillus spp.* are involved in biocontrol of soilborn pathogens. Strains of *Bacillus subtilis, Bacillus megatherium*, *Bacillus licheniforms* and *Bacillus pumilus* inhibit several phytopathogenic fungi, responsible for different diseases in plants with high economic value (55, 56, 57, 58, 59).

Bradyrhizobium japonicum, thought it fixes atmospheric nitrogen, it may contribute by other means in plant growth. *B. japonicum* and *A. brasilense* are able to promote germination and seedling growth, either singled or combined of maize (*Zea mays* L.) and soybeans (60).

I.5 - Root Exudates

Soil proprieties such as pH, nutrient availability are important for microbial community structure and survival. Plant roots release a wide range of compounds, including sugars, ions, vitamins, amino acids, purines and nucleosides to the soil and consequently, these compounds are exploited by soil bacteria (61).

Plants deposit up to 40 % of the photosynthetically fixed carbon via their root system in a processed named rhizodeposition, and there it becomes accessible for organisms in the rhizosphere (62). This causes a growth in the microbial population in the rhizosphere, and that

community composition is considerably distinct from the surrounding bulk soil, a phenomenon called the rhizospheric effect (63).

To reach and use these nutrients, bacteria need to have certain traits like motility, chemotaxis, attachment (61), to proceed to root colonization, which is an important step either for phytopathogenic or benefitial bacteria. Plants can produce molecules excreted to the rhizosphere via root exudates, and may select distinct microbial communities, depending on the molecules that constitute the exudates (64). Exudates can act as chemotatic signal containing molecules that mimics Quorum sensing signals, attracting PGPRs, and inhibiting pathogenic bacteria, trough niche competition, or antibacterial compounds (Fig. 3). Therefore plants can interfere with Quorum sensing controlled processes such as biofilm formation (62).

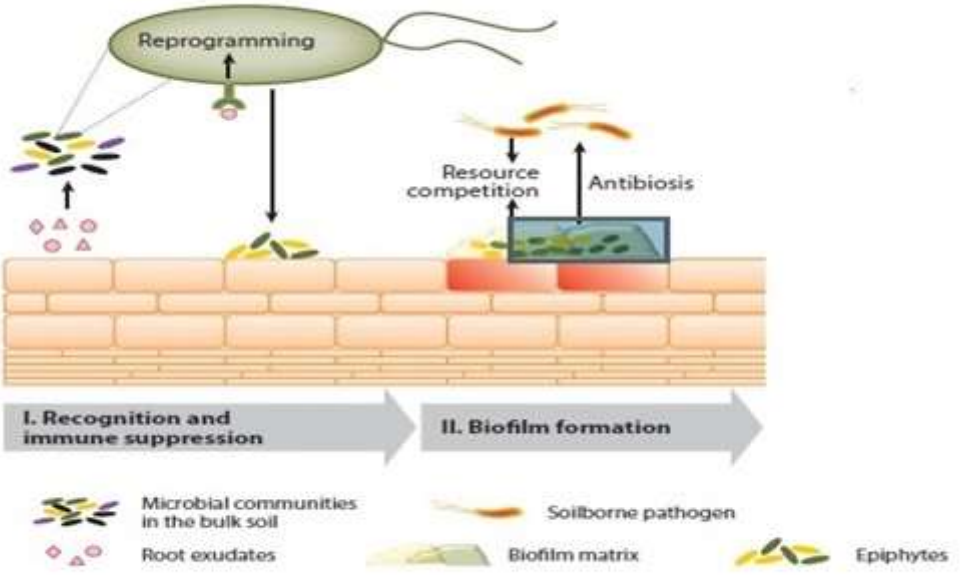


Fig. 3: Scheme of root colonization beneficial bacteria and their functions. (I) Plant roots exudate compounds that act as chemotactic signal. Some bacterial strains from the soil communities respond specifically to the signals and start express traits related to root colonization. Local immune responses in host roots are suppressed by PGPR. (II) PGPR on the root surface starts biofilm formation. PGPR may protect root tissues against pathogens via the production of antibiotics or niche and nutrient competition. Adapted from (65).

I.6 - Bacterial Inoculants

Bacterial inoculation emerges as an interesting alternative to chemical compounds in agriculture like pesticides, herbicides and fertilizers (66). The usage of such products has hazardous environmental effects, polluting the soil and ground water (67). These inoculations

have a wide range of utility, like biofertilization with nitrogen-fixing and phosphate solubilizer bacteria; phytohormone stimulation or production and last but not less important function as biocontrol agent protecting plants against pathogens (66).

So, the use of bacterial inoculants can have a direct effect on plant growth and protection, but it can also indirectly influence plants by influencing bacterial community of the rhizosphere, temporarily or permanently (66).

I.6.1 - *A. brasilense* inoculation

Inoculations with *A. brasilense* affect mostly plant performance and rhizosphere microbiome structure and function, and have already been successful in crop fields as maize among others (68). Studies have shown that effects on native microbial community vary according with *A. brasilense* strain and plant species. For instance, the strain Sp245 does not have much effect on bacterial community in rhizosphere with maize (69). Other strains as 40M and 42M change physiological profiles (CLPPs) of microbial communities of Asian rice (*Oryza sativa* L.) (70).

I.6.2 - Co-inoculation

Inoculation with more than one strain, have the purpose of getting a greater positive effect expecting those strains to act synergistically. This can be attempted with bacteria that in mono-inoculation have distinct effects. Nevertheless, competitive results between strains in inoculation may happen, consequently having reduced or even null effect on plants or in the rhizosphere community (66).

As example, co-inoculation of *B. subtilis* 101 with *A. brasilense* Sp245 neither had synergetic effect on plant biomass compared to their mono-inoculations in tomato (*Lycopersicon esculentum)* nor had significant effect in bacterial community in the rhizophere (71). Obtaining successful co-inoculations with synergetic effects of the bacteria represent a difficult task, thought already several have been discovered. As an example, co-inoculation of *Pseudomonas chlororaphis* IDV1 *and P. putida* RA2*,* is capable of diminishing the gram-positive dominant community and increasing the gram-negative community (72). Co-inoculation of DAPG-producing strain *P. fluorescens* F113 and *A. brasilense* Sp245-Rif in wheat resulted in enhanced root diameter, total root volume, and root number when compared to single

inoculations (40). There is research of *A. brasilense* co-inoculation with AMF proving it can be successful, like *A. brasilense* Az39 and *Glomus intraradices* improving rice growth in well watered conditions and in drought stress (73). *A. brasilense* Sp7 co-inoculated with either *Glomus clarum* or *Glomus fasciculatum* have a stimulant effect, increasing weight and root length of tomato (74). *A. brasilense* Az39 co-inoculated with *B. japonicum* E109 promote seed germination and early seedling growth in corn and soybean (60).

I.7 - Thesis goals and strategy

A. brasilense has been produced as inoculant by some companies, and applied in the agriculture as a biofertilzer in some countries (67). Therefore, there is a great interest in gathering more scientific knowledge that improves *A. brasilense* efficacy as a PGPR.

A. brasilense needs a vast array of traits to colonize plant roots, including chemotactic response, and biofilm formation. (61) Fungal and bacterial interaction and possible biofilm formation stimulation were an interesting subject to test.

The purpose of this thesis was to evaluate whether and how rhizospheric microbial interspecific interactions, namely with root symbiotic AMF and rhizospheric bacteria, could influence and stimulate biofilm formation in *A. brasilense*. To achieve this goal the influence of (i) AMF was evaluated by testing root exudates from maize plants colonized with or without different AMF species (*Glomus mosseae, Glomus claroideum, Glomus intraradices* and *Gigaspora sp*.) on biofilm formation and chemotactic response, and (ii) of interspecifc bacterial interactions on biofilm formation by testing (a) bacterial supernatants from several soil borne strains (*Pseudomonas putida* X236, *Pseudomonas fluorescens* 20130311XA1, *Mesorhizobium loti* B29, *Bacillus pumilus* B7, *Bacillus subtilis* 168, *Bacillus licheniformis* B6, *Bacillus megaterium* GY-23 and B3, *Bradyrhizobium japonicum* and *Azotobacter chroococcum*.), (b) bacterial co-inoculation with *P. putida* and *B. japonicum* (species selection based on results obtained) in several frequencies, and (c) bacterial-influenced root exudates from maize plants inoculated with *A. brasilense*, *P. putida* and *B. japonicum*.

To test whether biofilm is an important feature to improve *A. brasilense* as a PGPR, its function as a phytohormone producer was evaluated by performing IAA quantifications in biofilm and planktonic cells, and then to inquire any difference of produced IAA for each DL-tryptophan supplementation by cell type.

II - Materials and Methods

II.1 - Bacteria and growth conditions

Bacterial strains, used in this work are listed: *A. brasilense* AMC B1, *M. loti* AMC B28, *B. subtilis* 168, *P. putida* X236, *P. fluorescens* 20130311XA1, *B. megaterium* GY-23, *B. japonicum*, *A. chrocorum*, *B. licheniformis* AMC B6, *B. pumilus* AMC *B7*. AMF inoculum was acquired to Symbiom, Ltd, and *B. licheniformis, M. loti, B. megaterium, B. pumilus* and *A. brasilense* strains were supplied by AMC Chemicals & Trichodex, SL. The other strains were isolated from work developed at Faculty of Sciences, University of Lisbon. Bacterial strains were grown in NB medium (75) at 28 °C and 160 rpm in an orbital shaker, except *for B. japonicum*, which was grown in YMB (76).

II.2 - Biofilm growth and quantification

In all assays biofilms developed in microtiter plates of 96 wells, which were involved in Parafilm M® and incubated for 5 days at 28 °C, in a growth chamber. The medium utilized to allow biofilm growth and possible stimulation was NB diluted to 1/10 of original concentration. Diluted NB was chosen after a trial to test if there was any significant difference between usage of NB, YMB and LB. After testing, no significant difference was detected in biofilm formation (one-way ANOVA, $p>0.05$). Then, NB dilution at 10 % was used.

In each well of the 96 well microtiter, 40 µl of root exudate or bacterial supernatant, 20 µl of NB and 130 µl of distilled water were added, and supplemented with 10 µl of NB; pre-grown culture of *A. brasilense* or of *A. brasilense* in co-culture with other bacterial strain at different proportions. Control wells without root exudates or bacterial supernatant were also performed. In each plate there was one well corresponding to the blank, in which only diluted NB and distilled water was added. The number of replicates varied between 3, 5, 8 or 10 according with the assay.

Biofilm formation was quantified at the end of the assays, using Crystal Violet (CV) (77) method with slight modifications. Briefly, 200 µl of PBS buffer (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.44 g/l, KH_2PO_4 0.24 g/l, pH=7.4) was added drop-wise to wash the wells, then PBS was removed, and 200 µl of methanol was added. After 15 minutes, methanol was removed by letting the microtiter plate dry upside-down on tissue paper. Subsequently, the biofilms became fixed onto the wells surface, and 200 µl of CV 1 % (wt/vol) was added. Fifteen minutes later, the

excess of CV was removed by rinsing carefully the wells with tap water. Microtiter plates were dried upside-down on tissue paper, and then to dissolve attached stained cells from the walls, 200 µl of acetic acid 33 % (vol/vol) was added. Finally, 160 µl of each well were transferred to another sterilized microtiter plate.

For biofilm quantification, *A. brasilense* biofilm over walls of wells was measured by reading the optical density (OD) at 570 nm wavelength in a microtiter reader (Tecan Spectra Rainbow 1000). Planktonic cells together with the pellicle were transferred to another microter plate and quantified by reading OD at 600 nm wavelength. Both measurements were used to calculate later the Specific Biofilm Formation (SBF), formulated as SBF=(B-C)/G, where B is the OD_{570} of the attached biofilm cells on wells, C is the OD_{570} of the stained control wells, and G is the OD_{600} of the transferred planktonic cells (78, 79).

II.3 - Root exudates assay

II.3.1 - Plant growth of mycorrhized roots experiment

Maize was the plant chosen for the experiments. Seed sterilization were accomplished by putting the seeds in 70 % ethanol during 3 minutes, right after they were transferred to sodium hypochlorite 5 % (vol/vol) solution for another 3 minutes, and then the seeds were washed 5 times in sterilized distilled water. Seed germination was done in Petri dishes with filter paper on the bottom and involved around with adherent pellicle. Incubation of seedlings lasted 10 days in a dark chamber at 28 ^{o}C. Every 2 days some sterilized water was added to the Petri dishes, in the flux chamber.

Maize plants were put in 1.5 l pots with a substrate composed by 1 l of sterilized river sand and Styrofoam spheres (1:2). Plants were inoculated with 15 ml corresponding to 14 000 propagules of AMF inoculum per pot of *G. mosseae, G. claroideum, G. intraradices* and *Gigaspora sp*., except the controls where inoculum was not added. The plants were grown in a growth chamber at (25 ^{o}C, 75 % of relative humidity, 16 h light period). Plants were watered 3 times per week with Hoagland solution ½ (80) and water during the 45 days of development. Each treatment had 3 replicates.

II.3.2 - Plant growth of bacterial roots experiment

Maize was the plant chosen for the experiments. Seed sterilization and seedling growth was processed the same way as described previously in plant growth of the mycorrhized roots experiment.

Then plants were put in a sterile system (Fig. 4), composed by UNICORN® bags with 0.2 µm membrane allowing gas exchange, and polystyrene tubes and 3 of these systems were used. 1 ml of previously grown *A. brasilense*, *B. japonicum* or *P. putida* X236 until early exponential phase (OD_{600} of 0.12-0.15), were inoculated in the polystyrene tubes filled with 10 ml of NB medium. Later the plants were put in a growth chamber (25 °C, 75 % humidity, 16 h light period) and grew for 15 days.

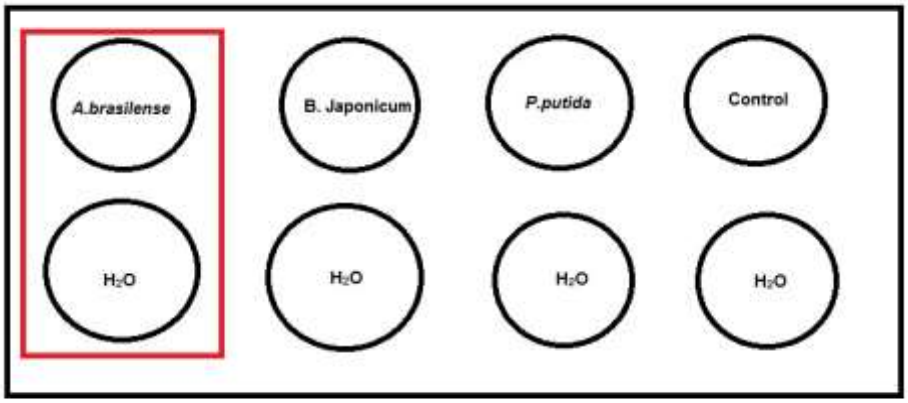


Fig. 4: Scheme of plant growth in the sterile system. The plastic bag is the outer rectangle, allowing sterility for plant growth. The circles represent the polystyrene tubes, the ones with H_20, were filled with water, and plant roots exudated there. The circles with bacterial strains names were the ones with NB inoculated with *A. brasilense*, *B. japonicum* and *P. putida* X236, the control circle was just filled with NB. The red rectangle represents the tubes in which roots of the same plant were immersed in NB inoculated with bacteria and immersed in water.

II.3.3 - Root Exudates preparation

After plant growth, to collect root exudates from the bacterial experiment, plants were then removed from the tubes, and the water where maize roots had been exudating was filtrated through Ø 0.22 µm nitrocellulose filters (Millipore®) and solutions were kept at -20 °C until use. Collection of the root exudates from mycorrhized plants was similar; except that root system was incubated in Erlenmeyer flasks filled with 100 ml sterilized Milli-Q water for 24 h (81).

II.3.4 - Biofilm Formation evaluation

A total of 3 microtiter plates with each one having 3 replicates of every sort of root exudate with and without inoculation (*A. brasilense, B. japonicum and P. putida* X236) were used, being each replicate a sample of each plant growth system.

The assay with root exudates from mycorrhized maize plants (*G. mosseae, G. claroideum, G. intraradices* and *Gigaspora sp*.), was performed in the same way.

II.4 - Chemotaxis assay

Chemotaxis assay (82) was performed using the "drop" assay with slight modifications, with the purpose to test the effect of root exudates collected from mycorrhized maize plants, and compared to some organic acids (malate, succinate) and amino acids (L-aspartate, L-serine, L-arginine, L-glycine, L-proline, L-cysteine, L-glutamine) normally found in maize root exudates.

Briefly, *A. brasilense* grown 48h in NB was diluted 100-fold into 150 ml of NB. When *A. brasilense* reached the early exponential phase (OD_{600} of 0.12-0.15), 40 ml samples were centrifuged (Eppendorf centrifuge 5810R) at 3 000 rpm for 10 minutes and re-suspended in 12 ml of chemotaxis buffer (100 mM potassium phosphate, pH 7.0, 20 µM EDTA). An aqueous solution of 1 % hydroxypropylmethylcellulose (Sigma-Aldrich), formulated to give a viscosity of approximately 4 000 cP in a 2 % aqueous solution, was added to the cell suspension to give a final volume of 15 ml. The resulting suspension was transferred to a Petri dish. In the Petri dish center, 10 µl of each root exudate, organic acid and amino acid were dropped. After 2 hours of incubation at room temperature, the plates were analyzed for the appearance of a clear zone, and measured. The bacteria attracted by the added chemoattractant leave a clear zone where they were previously sited. Three replicates were used for each compound and their concentration was 40 mMol.

II.5 - Bacterial supernatant assay

II.5.1 - Supernatant preparation

Bacterial strains of *P. putida* X236, *P. fluorescens* 20130311XA1, *M. loti* B29, *B. pumilus* B7, *B. subtilis* 168, *B. licheniformis* B6, *B. megaterium* GY-23 and B3, *B. japonicum* and *A.*

chroococcum, previously grown until late exponential phase (OD_{600} of 1.0-1.5) were centrifuged (Eppendorf centrifuge 5417C) at 13 000 rpm for 10 minutes, and then the supernatants were collected. For further analysis two bacteria were selected, *P. putida* X236 and *B. japonicum*. Supernatant preparation was performed identically.

II.5.2 - Biofilm formation evaluation

A total of 3 microtiter plates with each one having 5 replicates for each supernatant treatment and control were used.

II.6 - Co-innoculation assay

II.6.1 - Co-inoculation

P. putida X236 and *B. japonicum* were inoculated each one with *A. brasilense* in a pairwise co-culture system in several proportions, being *A. brasilense* in 0, 10, 25, 50, 75, 90 and 100 % frequency. Grown until OD_{600} of 1.0-1.5.

II.6.2 - Biofilm formation evaluation

A total of 3 Microtiter plates with each one having 5 replicates for each co-inoculation were used. In *A. brasilense-P. putida* X236 co-inoculations 2 controls were used: *A. brasilense*, *P. putida* X236 growing separately and in *A. brasilense-B. japonicum* co-inoculations 2 controls were used, but instead of *P. putida* X236, *B. japonicum* was used as control.

II.7 - Indol-3-acetic-acid quantification assay

IAA was quantified by using a modified Salkowski colorimetric assay (83, 84) in microtiter plates. Briefly, grown cultures of *A. brasilense* (early exponential phase OD_{600} of 0.12-0.15) were inoculated in microtiter plates. After they were grown for 3 days, planktonic cells were removed, 200 µl of NB 1/10 supplemented with DL-tryptophan (0, 10, 25, 50, 100, 200 mg/ml) was added. Two days later, 170 µl of each well was transferred to a new microtiter plate, then the plates

were centrifuged (Eppendorf centrifuge 5810R) at 3 500 rpm for 10 minutes. After centrifugation 150 µl of each well were transferred to a new plate, followed by addition of 100 µl of Salkowski reagent ($FeCl_3.6H_2O$ 0.5 M and $HClO_4$ 35 %). The reaction took 30 minutes in a dark chamber and right after microtiter plate reader (Tecan Spectra Rainbow 1000) was used to measure OD_{530}. A total of 3 plates were used, each one with 10 replicates of every DL-tryptophan supplementation and the control. The blank corresponded to one well of NB 1/10. Then IAA detected by Salkowski colorimetric assay in planktonic cells and biofilm were compared.

II.8 - Data analysis

The experiments conducted had a design in which every treatment was replicated 3 to 10 times in every microtiter plate depending on the assay. Average of replicates was calculated, and then each microtiter plate functioned as a single replicate of a total of 3 replicated plates. Statistical analysis of the data was done using Softstat *Statistica* 11® software following one-way analysis of variance (ANOVA), except for IAA quantification (see below); when the ANOVA was significant the means were separated using a *post-hoc* test, Tukey Honest Significant Difference (HSD) at $p \leq 0.05$ level of significance. Two-way ANOVA was utilized merely in IAA quantification assay testing the effects of cell type and DL-tryptophan concentration, and subsequently Student's *t*-tests were performed to inquire any significant difference between the cell number of biofilm and planktonic cells, as well in the differences of produced IAA for each DL-tryptophan supplementation by cell type. When necessary data was transformed by $\log_{10}(X)$, before proceeding to ANOVA.

III - Results

III.1 - Interactions with AMF

III.1.1 - Effects of root exudates from mycorrhized plants on biofilm formation

The results of the assay of effects of root exudates from mycorrhized maize plants on biofilm formation showed no significant differences between AMF treatments and control and among AMF species (Fig. 5), as confirmed by one-way ANOVA analysis ($F_{5, 12}$=1.574, p=0.240). Specific Biofilm Formation (SBF) had the same outcome (one-way ANOVA, $F_{5, 12}$=0.537, p=0.745) (Fig. 6).

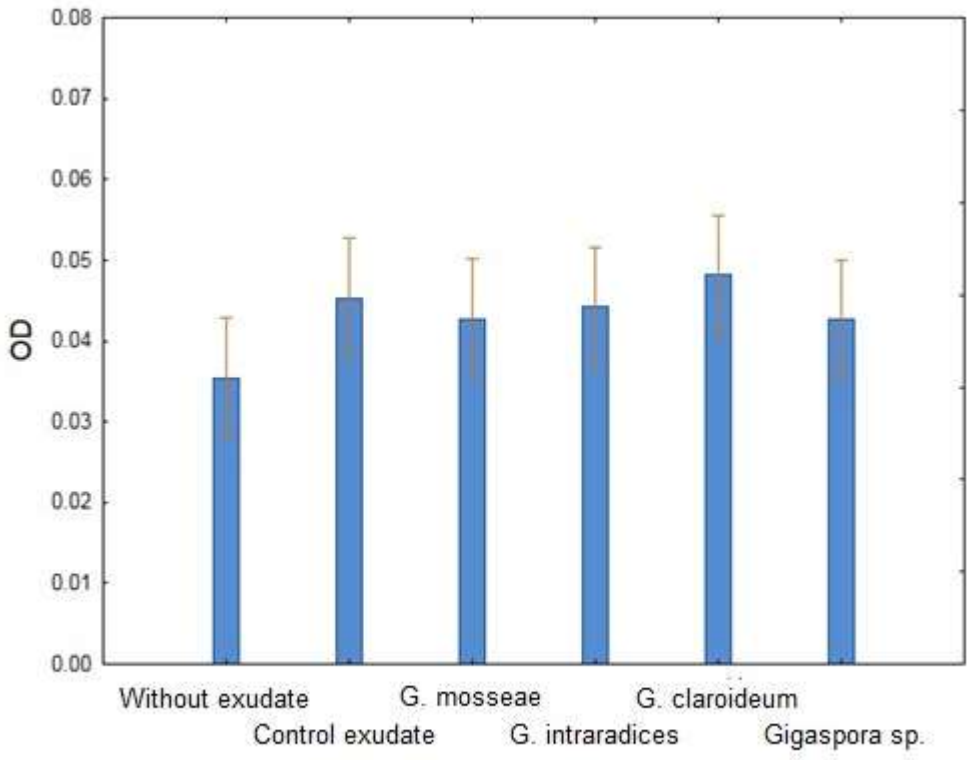


Fig. 5: *A. brasilense* biofilm quantification by CV method, from the assay of effects of root exudates from mycorrhized plants. Subtitles: OD, optic density; X-axis, root exudates origins. Error bars represent the standard deviation of the mean (n=3).

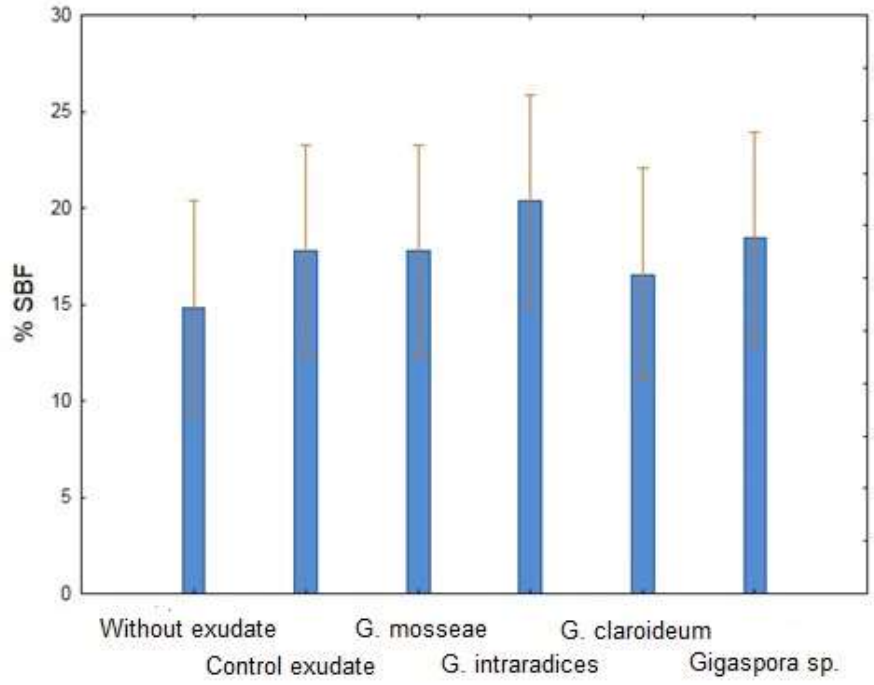


Fig. 6: One-way ANOVA of effects of root exudates from mycorrhized plants assay. Subtitles: % SBF, biofilm percentage given by Specific Biofilm Formation (SBF) ratio; X-axis, root exudates origins. Error bars represent the standard deviation of the mean (n=3).

III.1.2 - Effects of root exudates from mycorrhized plants on Chemotaxis

A. brasilense dropped in the middle of the Petri dishes, when attracted by either the exudates or other compounds added beforehand generated a clear zone. By other words, this zone corresponds to the migration of bacterial cells where they were previously sited to the Petri dish center. One-way ANOVAs, comparing organic acids and amino acids (Fig. 7) showed significant differences between treatments ($F_{8, 18}$=17.485, p<0.001), as in the comparison of root exudates (Fig. 8) ($F_{4, 10}$=97.750, p<0.001). Subsequently *post-hoc* Tukey HSD tests were performed.

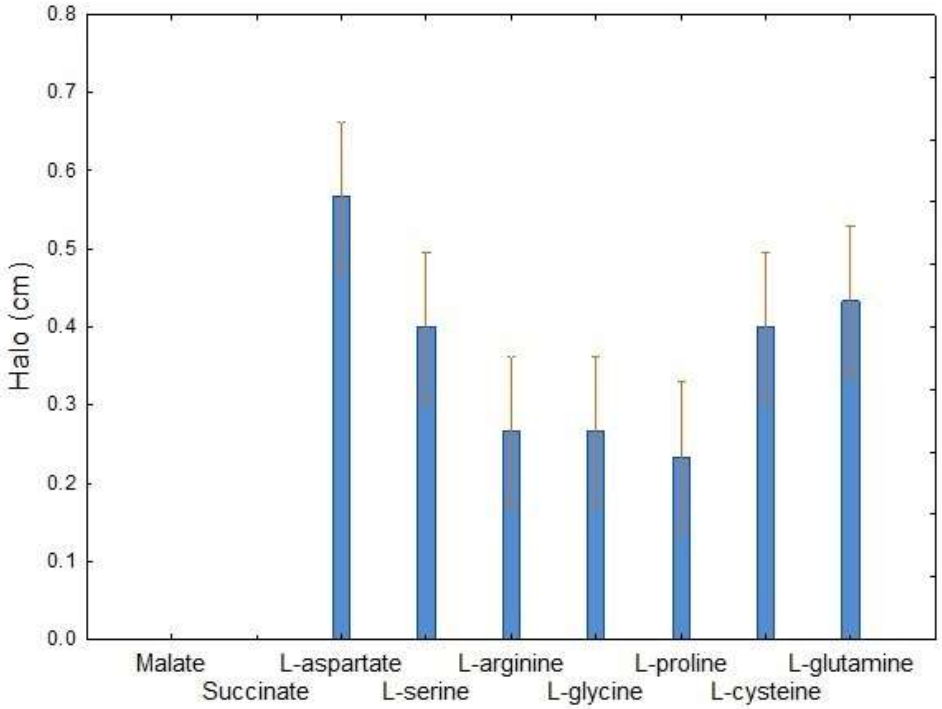


Fig 7: Chemotatic response of *A. brasilense* to several amino acids and organic acids. Control refers to root exudates from non-mycorrhized plants. Subtitles Halo (cm), halo in centimeters (clear area created by bacteria where they were previously sited); X axis, compounds used in the assay. Error bars represent the standard deviation of the mean (n=3).

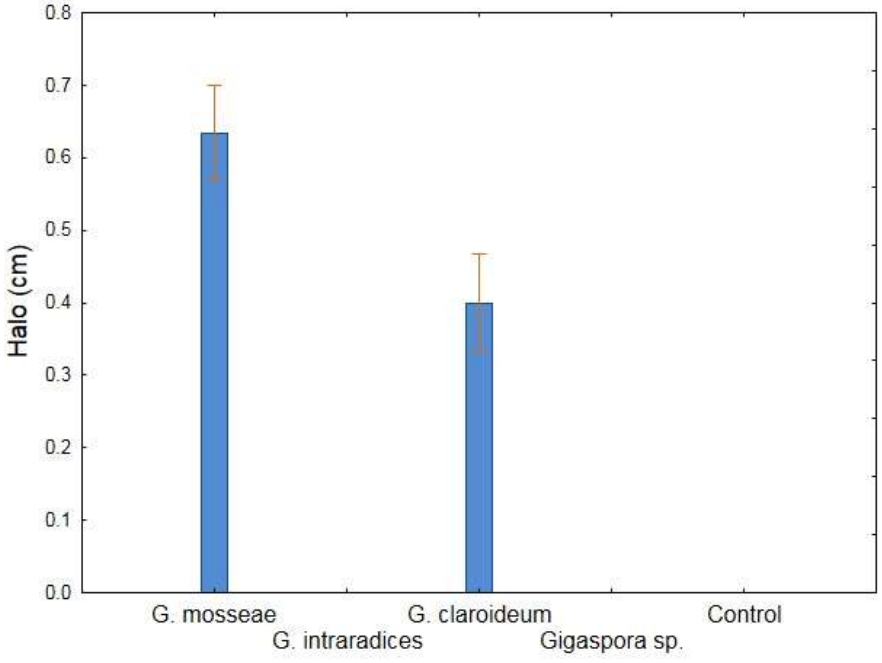


Fig 8: Chemotatic response of *A. brasilense* to several root exudates collected from mycorrhized plants. Control refers to root exudates from non-mycorrhized plants. Subtitles Halo (cm), halo in centimeters (clear area created by bacteria where they were previously sited); X axis, compounds used in the assay. Error bars represent the standard deviation of the mean (n=3).

Analyzing differences in the compounds tested (Fig. 7) it is perceptible that there are significant differences among the amino acids tested, comparing amino acids with L-aspartate significant differences can be found, namely L-arginine, L-glycine and L-proline. Examining root exudates (Fig. 8), three root exudates did not generate a chemotactic response (control, *G. intraradices and Gigapsora sp.*), whereas *G. mosseae* and *G. claroideum* did (p<0.05). Moreover, there was a significant difference between those exudates which created a chemotactic response, being *G. mosseae* root exudate the one who formed the largest clear zone in the Petri dishes.

III.2 - Interactions with bacteria

III.2.1 - Bacterial supernatants assay

A screening with several soil borne bacterial species was performed to assess which species stimulate *A. brasilense* biofilm, and whether there is a phylogenetic trend in inter-specifc interactions influencing the formation of biofilm. The results indicated that only the supernatant of the strain *P. putida* X236 stimulate biofilm quantity of *A. brasilense* compared to without bacterial supernatant (*t*-test, p=0.03), and all the other strain supernatants tested did not influence biofilm formation, that is, there were no significant differences (*t*-test, p>0.05) in the quantity of biofilm formed when compared to without supernatant (Fig. 9).

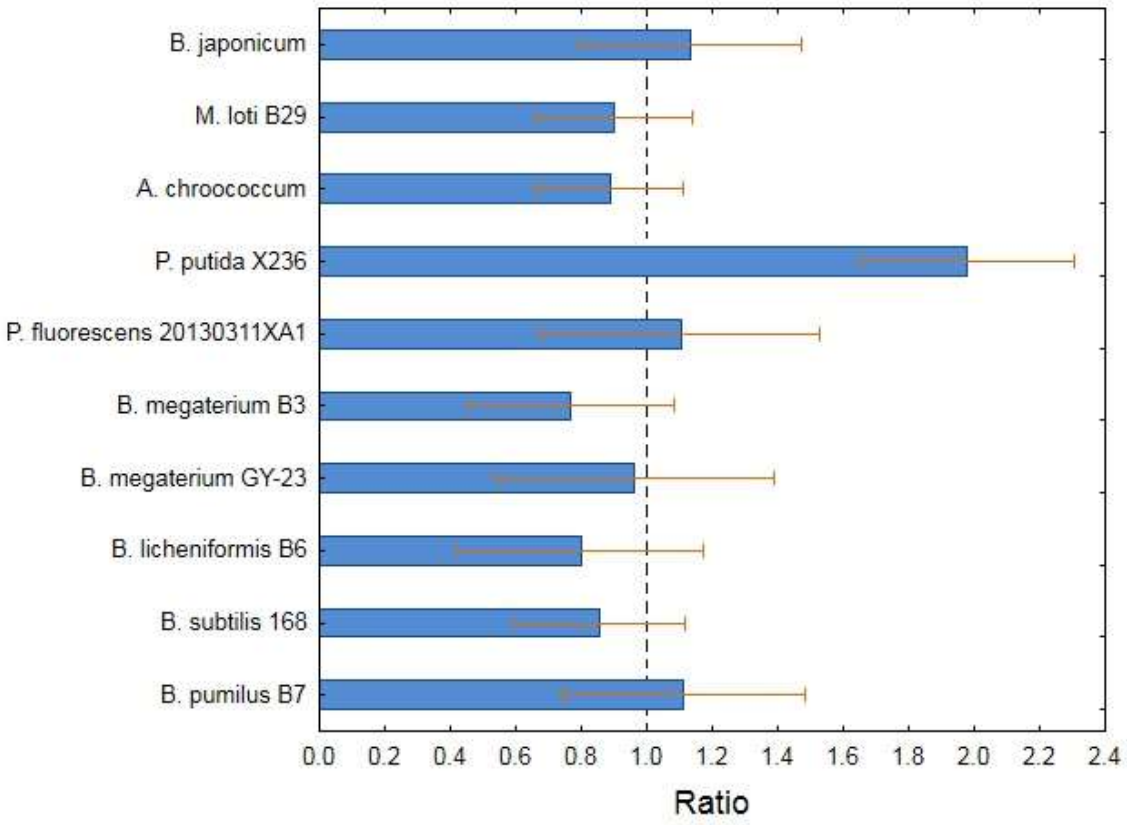


Fig. 9: Ratio of *A. brasilense* development according the supernatant treatment, from the screening of the assay of effects of bacterial supernatants from several strains. The ratio corresponds to (OD of *A. brasilense* grown with bacterial supernatant/OD *A. brasilense* grown without supernatant). The dashed line represents the growth of *A. brasilense without supernatant*. Subtitles: Y-axis, supernatant origins. Error bars represent the standard deviation of the mean (n=3).

P. putida X236 were then selected for further analyses and assays, together with *B. japonicum* as a bacterial strain control and because this nitrogen-fixer strain of *B. japonicum* was previously observed to stimulate root exudation and plant growth (60). The one-way ANOVA comparing *A. brasilense* biofilm quantification (Fig. 10) of these two strains with the control (without bacterial supernatant) showed significant differences ($F_{2, 6}$=9.567, p=0.013), whereas for SBF one-way ANOVA (Fig. 11) did not show any significant differences ($F_{2, 6}$=3.076, p=0.120).

Post-hoc Tukey HSD test performed in the biofilm quantification indicated that *P. putida* X236 presented significant differences when compared with either to control (without bacterial supernatant) or to *B. japonicum* supernatant. Despite similar results to biofilm quantification with highest value belonging to *P. putida* X236, the results of SBF were not statistically significant.

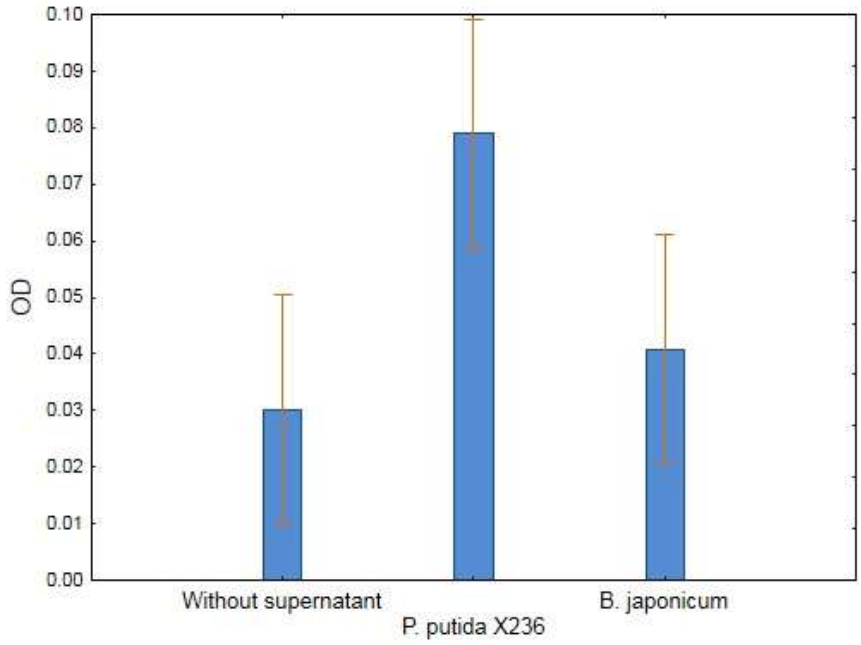


Fig. 10: *A. brasilense* biofilm quantification from the assay of effects of bacterial supernatants, using the CV method. Subtitles: OD, optic density; X-axis, supernatant origins. Error bars represent the standard deviation of the mean (n=3).

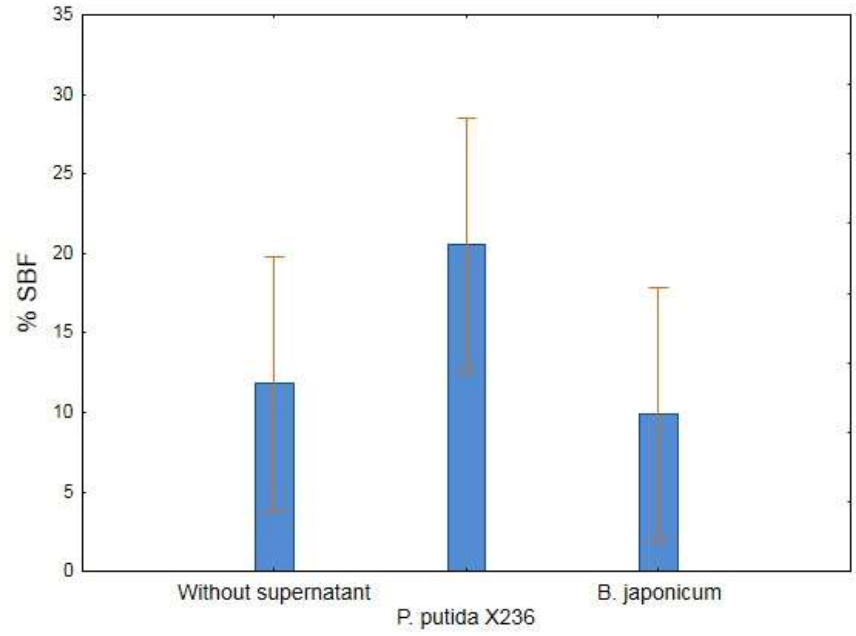


Fig. 11: *A. brasilense* SBF from the assay of effects of bacterial supernatants. Subtitles: X-axis, bacterial supernatant origin; % SBF, biofilm percentage given by Specific Biofilm Formation (SBF) ratio. Error bars represent the standard deviation of the mean (n=3).

III.2.2 - Co-inoculations assay

The co-inoculation assay was composed by two experiments. In the first one, a co-inoculation experiment of *A. brasilense* and *P. putida* X236, the one-way ANOVA (Fig. 12) demonstrated significant effects of treatment ($F_{6, 14}$=7.733, p=0.008). Consequently *post-hoc* Tukey test was performed to inquire the significant differences of biofilm formed in different settings.

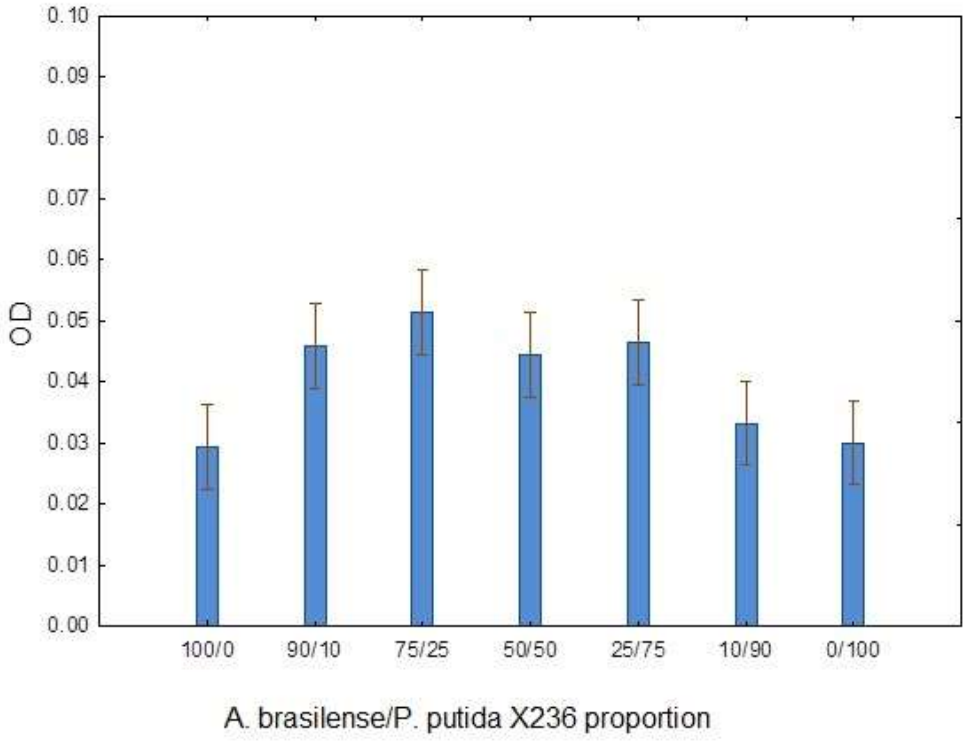


Fig. 12: Biofilms quantification, by CV method, from the co-inoculations of *A. brasilense* and *P. putida* X236 experiment testing different initial frequencies. Subtitles: OD, optic density; the ratio (X/Y) in which X is *A. brasilense* and Y is *P. putida* X236 initial inocula frequencies. The numbers in (X/Y) are *A. brasilense* / *P. putida X236* proportion. Error bars represent the standard deviation of the mean (n=3).

Both controls (single strain inoculation) showed significant differences with the co-inoculations in the proportion of 90/10, 75/25 and 25/75, having a lower OD and therefore less biofilm formed. Co-inoculation 75/25 also showed a significant difference when was compared to 10/90. All other co-inoculations did not display any significant difference when compared between themselves. With this result it is perceptible that the *A. brasilense* / *P. putida* X236 initial frequency of 75/25 is the co-inoculation which creates the highest OD value, hence the highest biofilm quantity. *A. brasilense* - *P. putida* X236 co-inoculations had encouraging results,

and SBF ratios as well (Fig. 13). All co-inoculations had higher SBF ratios than both single strain inoculations, with 90/10 (*A. brasilense* / *P. putida* X236) with the highest ratio. Also as the bacterial proportion in the co-inoculations increases in favor to *P. putida* X236 and *A. brasilense* lowers, the SBF ratio had a declining tendency (ANOVA, $F_{6, 14}$=5.282, *p*=0.049). Although, in these co-inoculation experiments it was not possible to distinguish between cells of *A. brasilense* and the other species tested in the formed biofilm, the declining biofilm formation (as SBF) with decreasing initial frequency of *A. brasilense* (Fig. 12, 13) suggests that *A. brasilense* biofilm was influenced by *P. putida* X236 when in co-culture, as it was found in the supernatant assay (Fig. 9, 10).

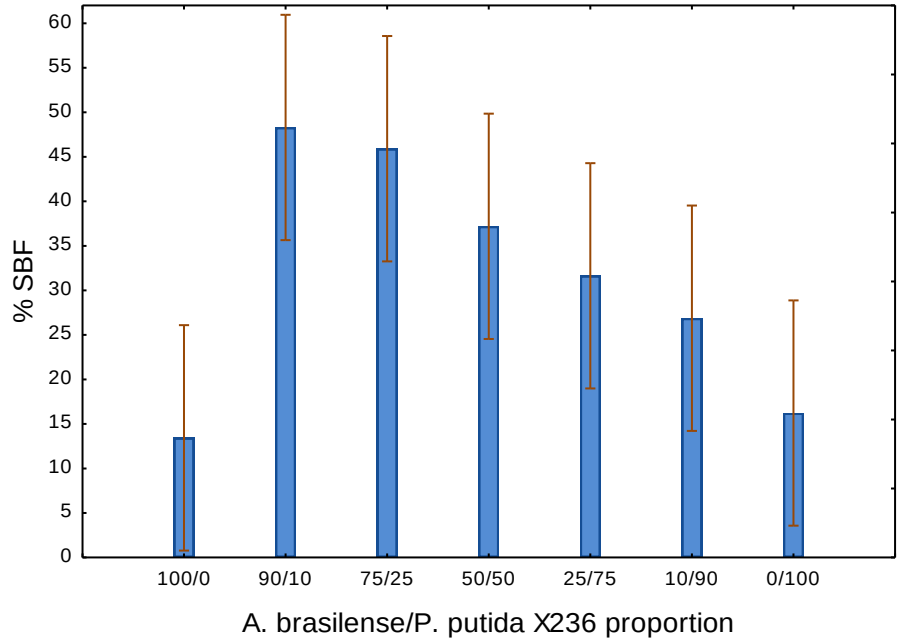


Fig. 13: SBF from the experiment of co-inoculations *A. bransilense* - *P. putida* X236. Subtitles: the ratio (X/Y), in which X is *A. brasilense* and Y is *P. putida* X236 initial inocula frequencies. The numbers in (X/Y) are *A. brasilense* / *P. putida* X236 proportion. %, biofilm percentage given by Specific Biofilm Formation (SBF) ratio. Error bars represent the standard deviation of the mean (n=3).

The other experiment, in which *A. brasilense* and *B. japonicum* co-inoculation was evaluated, had a different outcome. After $\log_{10}$(X) transformation, one-way ANOVA did not show significant effects ($F_{6, 14}$=0.610, *p*= 0.718), which indicates that there is no significant difference in the biofilm formed, independently of the co-inoculations proportions (Fig. 14).

The one-way ANOVA of SBF ratio for *A. brasilense-B. japonicum* co-inoculation (Fig. 15) had a significant result ($F_{6, 14}$=3.954, *p*=0.015), and Tukey HSD tests indicated that only the frequency of 50/50 made a significant difference compared to both single strain inoculations.

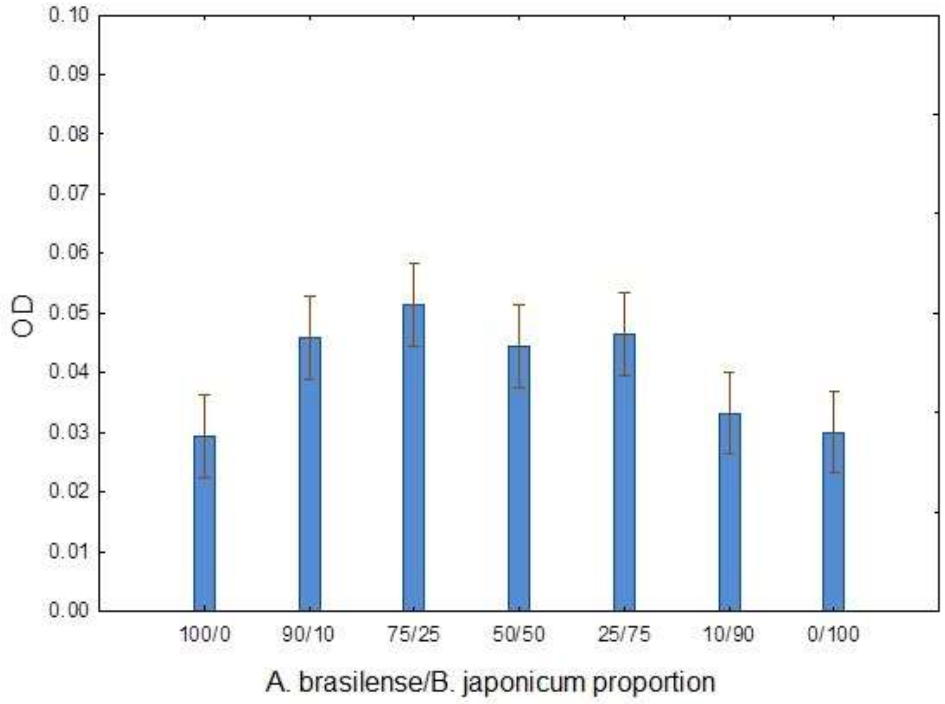


Fig 14: Biofilms quantification, by CV method, from the co-inoculations of *A. brasilense* and *B. japonicum* experiment testing different initial frequencies. Subtitles: OD, optic density; the ratio (X/Y) in which X is *A. brasilense* and Y is *B. japonicum* initial inocula frequencies. The numbers in (X/Y) are A. brasilense / *B. japonicum* proportion. Error bars represent the standard deviation of the mean (n=3).

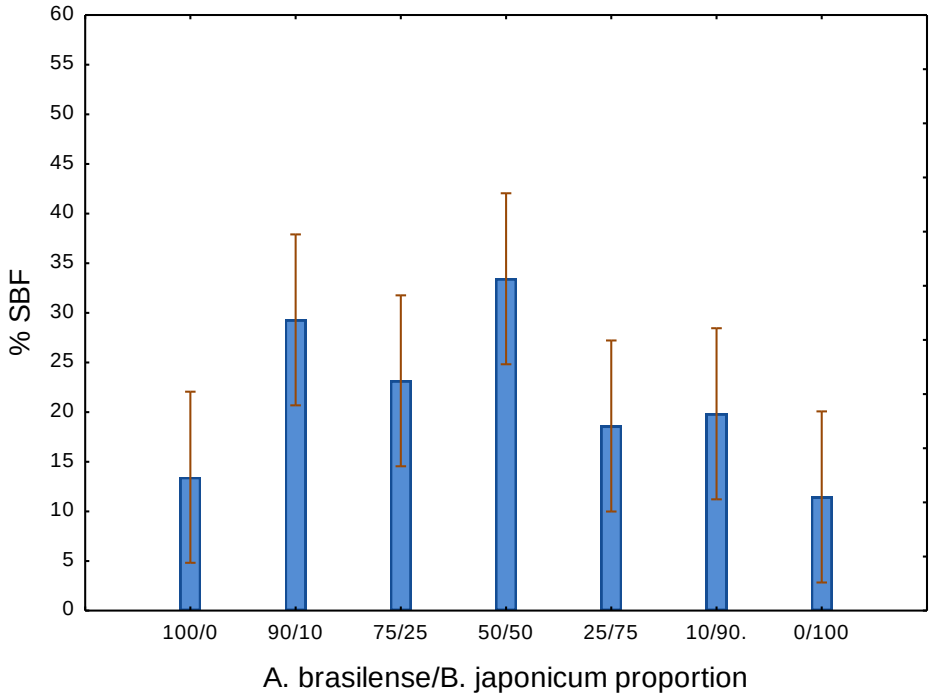


Fig. 15: SBF from the experiment of co-inoculations *A. bransilense* - *B. japonicum*. Subtitles: (X/Y) is the ratio, in which X is *A. brasilense* and Y is *B. japonicum* initial inocula frequencies. The numbers in (X/Y) are A. brasilense / *B. japonicum* proportion. % SBF, biofilm percentage given by Specific Biofilm Formation (SBF) ratio. Error bars represent the standard deviation of the mean (n=3).

III.2.3 - Effects of *A. brasilense, P. putida* X236 and *B. japonicum* via root exudates assay

In this assay testing the indirect effects of bacterial strains in *A. brasilense* biofilm formation *via* maize root exudates, ANOVA results showed that root exudates effects were significant ($F_{4, 10}$=8.109, *p*=0.003), with significant differences (Tukey HSD test) in biofilm formation between root exudates from planta inoculated with *A. brasilense* and without exudate, control exudate or *P. putida* X236 (Fig. 16). Therefore, there was a significant positive influence of root exudates from plants inoculated with *A. brasilense*, but not with *P. putida* X236 or *B. japonicum*.

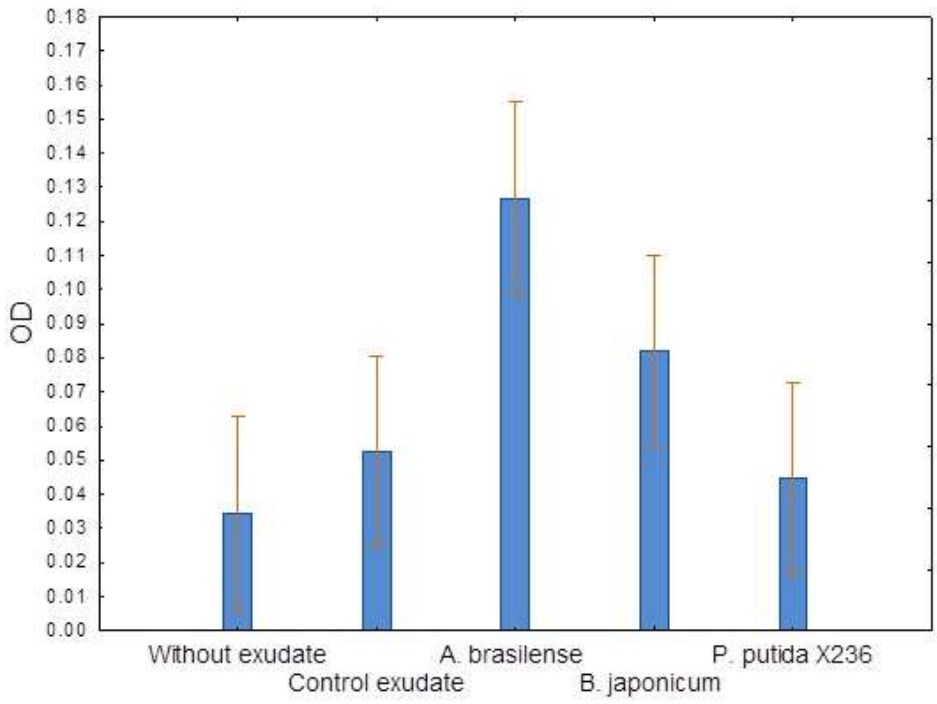


Fig. 16: *A. brasilense* biofilm quantification by CV method from the assay of effects of root exudates from plants inoculated with *A. brasilense, P. putida* X236 and *B. japonicum.* Subtitles: OD, optic density; X-axis, root exudates origins. Error bars represent the standard deviation of the mean (n=3).

For root exudates effects on SBF, one-way ANOVA also showed significant results ($F_{4, 10}$=8.207, *p*=0.003), with significant increases in SBF (Tukey HSD test) of the root exudates from plants inoculated with *A. brasilense* or *B. japonicum* compared to without exudate (Fig. 17). Root exudates from plants with inoculated bacteria did not have any significant difference compared to control exudate.

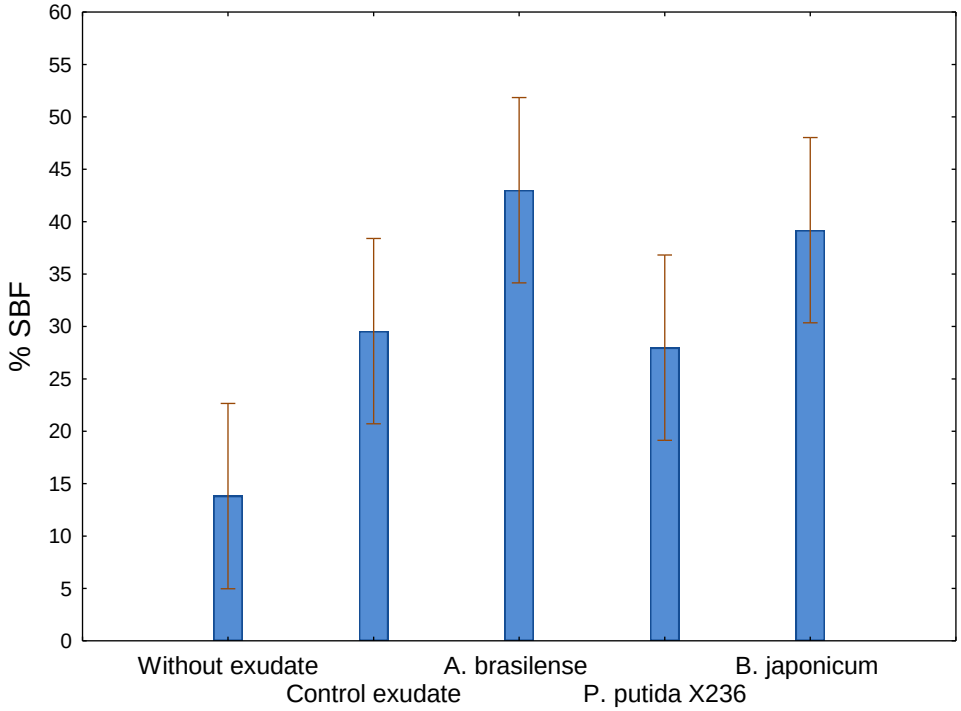


Fig. 17: SBF from the assay of effects of root exudates from plants inoculated with *A. brasilense, P. putida* X236 and *B. japonicum.* Subtitles: %, biofilm percentage given by Specific Biofilm Formation (SBF) ratio; X-axis, root exudate origins. Error bars represent the standard deviation of the mean (n=3).

III.3 - Indol-3-acetic-acid quantification assay

IAA quantification assay was performed, to confirm the capacity of the *A. brasilense* to synthetize this phytohormone, and to inquire any noticeable differences in the amount formed in biofilm or planktonic lifestyle (Fig. 18). Two-way ANOVA showed that the main effects were all significant ($F_{1, 48}$=145.753, p<0.001 for cell lifestyle; $F_{5, 48}$=699.904, p<0.001 for DL-tryptophan concentration; $F_{5, 48}$=165.342, p<0.001 for factor interaction). Subsequently *t*-tests were performed to inquire any significant difference among cell number of biofilm and planktonic cells, also this test served to investigate if there was any significant difference of produced IAA for each DL-tryptophan supplementation by cell type. Difference in cell number amongst biofilm and planktonic was significant (p=0.045), being the highest values corresponding to planktonic cells. Without DL-tryptophan supplementation the IAA produced is 26.5 and 10.5 µg/ml in biofilm and planktonic form respectively, and *t*-test result showed significant differences (p=0.045). As the supplementation increases IAA quantified raises too. For 10, 25 and 50 µg/ml DL-tryptophan supplementations, biofilms produced significantly more IAA than planktonic cells (p<0.001). At 100 µg/ml supplementation IAA quantity was similar, around 300 µg/ml and no

significant differences were detected (p=0.910). At 200 µg/ml supplementation planktonic cells produced significantly more IAA than biofilms (p<0.001).

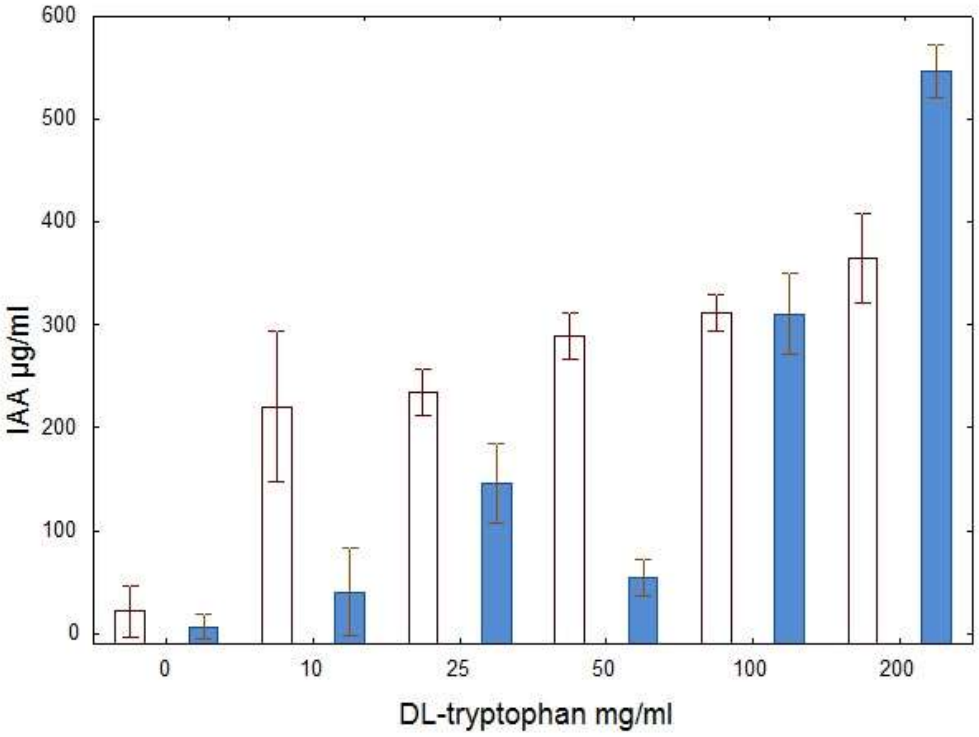


Fig. 18: IAA quantification of *A. brasilense* biofilms and planktonic cells, as white and blue bars respectively, by a modified Salkwoski colorimetric method in microtiter plate. Subtitles: the numbers on the x-axis represent the DL-tryptophan concentration in mg/ml; IAA µg/ml is the amount of IAA synthesized detected by the colorimetric method. Error bars represent the standard deviation of the mean (n=3).

IV - Discussion

IV.1 – Microbial Interactions

The several assays conducted allowed the thesis goals achievement. The influence of other microorganisms in *A. brasilense* biofilm formation was evaluated either directly or indirectly. In the first case this was attained by using bacterial supernantats of several rhizospheric bacteria. In the second case, root exudates were used whether from maize plants colonized with AMF (*G. mosseae, G. claroideum, G. intraradices* and *Gigaspora sp*.) or colonized by *A. brasilense*, *B. japonicum* and *P. putida* X236. Also, AMF were used to test chemotactic response.

Regarding interspecific bacterial interactions impact on biofilm formation, bacterial supernatants from several soil borne strains were used. The bacteria were chosen due to their importance as PGPR and wide range of biological activity in the rhizosphere. Moreover, *P. putida* X236, *P. fluorescens* 20130311XA1 were selected due to Pseudomonads biocontrol capabilities (51, 52, 53, 54). Not only, but also species of *Bacillus* have those proprieties (55, 56, 57, 58, 59). Thus, strains of *B. pumilus*, *B. subtilis*, *B. licheniformis* and *B. megaterium* were selected. In addition, due to their nitrogen fixation strains of *B. japonicum, A. chroococcum* and *M. loti* (85, 86, 87) were picked too. Considering the supernatants of the selected bacteria, *P. putida* X236 had a positive and significant effect on *A. brasilense*. After examining the results, there was no link with the bacteria phylogeny. As consequence of the screening and posterior supernatant influence in biofilm formation, was provided further focus on *P. putida X236*, which showed more potential. Therefore, *P. putida* X236 had a direct effect in *A. brasilense* biofilm formation.

These findings were complemented by the co-inoculations assays, in which *P. putida* X236 along with *A. brasilense* made a worthy co-inoculation when *A. brasilense* was in higher initial proportion, compared to *P. putida* X236 in the inoculum. In addition, the higher Specific Biofilm Formation ratios were present when *A. brasilense* was the dominant presence in relation to *P. putida* X236, and as the bacterial proportion in the co-inoculations increases in favor to *P. putida* X236, the SBF ratio has a declining tendency, leading to the corroboration that it is *P. putida* X236 that is influencing *A. brasilense* and not otherwise. Cell number of each strain in the inoculant is unknown, but these results suggest that *P. putida* affects *A. brasilense* biofilm formation when in co-inoculation, possibly synthetizing a signaling molecule. Likewise a stable and successful co-inoculation is presented. Both bacterial species are known for their role as PGPR, *P. putida* acting as a biocontrol agent (51, 52), and *A. brasilense* as nitrogen fixer and

phytohormone producer (23, 24). However, studies about *P. putida* X236 were not found in the literature. Nevertheless, it is a good begin for a future co-inoculant consortium. Field tests would be necessary as it is already been shown in several studies that *in vitro* and *in vivo* (crop field) results are sometimes different (66) and to ensure a synergism of the strains in inoculant is accomplished. *P. putida* strains have the potential to even shift the microbial communities from being, dominant gram positive to gram negative (70).

In relation to indirect effects in biofilm formation by *A. brasilense,* specifically via root exudates, neither *P. putida* X236 nor *B. japonicum* had pronounced influence. Nonetheless, maize root exudates colonized of either AMF or PGPR, produced interesting results. Exudates from plants inoculated with *A. brasilense* influenced biofilm formation of itself, whereas exudates from mycorrhized plants only had effects *A. brasilense* chemotaxis. Moreover, *G. mosseae* created a greater chemotactic response by *A. brasilense*. Both microorganisms are recognized to stimulate maize to synthetize several cyclic hydroxamic acids, which may accumulate in roots and are known for their beneficial effect on plants immune response, namely against fungal and bacterial pathogens. One of those compounds is 2, 4-dihydroxy-7-methoxy-2H-1, 4-benzoxazin-3(4 H)-one (DIMBOA). In addition, *A. brasilense* depending on the strain and maize cultivar may impact secondary metabolism, either stimulating DIMBOA synthesis or 6-methoxbenzoxazolin-2-one (MBOA), thereby these secondary metabolites may be exudated by roots and even in a larger scale in plants inoculated with *A. brasilense* (88, 89).

Despite of biocontrol capabilities of these molecules, recent research has shown *that P. putida* KT240 may be attracted by DIMBOA to the maize roots. Furthermore, studies demonstrated that at least in wheat, DIMBOA and MBOA may actually alter microbial communities in the rhizospere, increasing Gram-negative bacteria biomass (90, 91). Even though this should be taken carefully, as DIMBOA usually degrades quickly in soil into its derivates such as MBOA (92). Based in the literature, it could be hypothesized that in the present study *A. brasilense* may had stimulated DIMBOA synthesis and exudation by the roots and trigger biofilm formation. Regarding exudates from plants inoculated with *G. mosseae*, they could contain DIMBOA, which might had generated a chemotactic response by *A. brasilense*.

Regarding the organic acids utilized, this strain of *A. brasilense* did not generate chemotactic response towards those organic acids. This results are according to previous studies, which showed that chemotaxis in *Azospirillum* is strain dependent (32). As for amino acids results were consistent as previous studies describe, *A. brasilense* is attracted towards amino acids commonly found in root exudates (93) and are used as a nitrogen source. However, amino acid composition may be different among plant species and change throughout their

development, also as mentioned in previous research, specific amino acids may have a major role in colonization of a specific plant species and for nitrogen fixation as well (31).

Summing up, root exudates assays aided to verify that certainly *P. putida* X236 has a direct stimulating effect in *A. brasilense* biofilm formation, which is not through maize root exudates.

IV.2 – IAA

The IAA production detected raises either in biofilm as in planktonic cells. This is expected due to several tryptophan dependent pathways like Indole-3-pyruvate pathway, being this one thought to be one of the major pathway for IAA biosynthesis (94). In the absence of tryptophan supplementation, IAA synthesis is viable due to tryptophan independent pathway, which was demonstrated in *A. brasilense* (94).

Looking at the results, in biofilm IAA was detect in greater concentrations without or with low supplementation compared to planktonic cells, and the last ones reach their uppermost synthesis of IAA with high supplementation, consequently is conceivable to claim that in biofilm *A. brasilense* is less dependent on tryptophan as precursor for IAA synthesis, using tryptophan independent pathway to synthetize IAA, and while as free living this bacteria is more prone to utilize tryptophan dependent pathways. However, these results should be analyzed carefully since the difference in cell numbers are significant, being higher in planktonic cells, which might possibly caused the difference in IAA synthesis in the assay. In addition, there is in course a reviewing process of the independent tryptophan pathway as until now no specific enzymes of this pathway have been described (94). Since *A. brasilense* colonizes the roots, being this region, especially the tip of the root rich in amino acids (95), with a large support of amino acids, it was expectable that in biofilm the usage of tryptophan pathways was more dominant.

V - Concluding Remarks

Comprehend what may influence *A. brasilense* development and function is crucial in such high potential bacteria to be used in agriculture as a biofertilizer, contributing to reduce pollutions by in the future being a substitute to chemical fertilizers. Increasing biofilm formation might help to reduce the costs of a future inoculum and that way encourage its utilization. Therefore it is important to study how biotic factors influence biofilm formation and chemotaxis of this species.

With this work it was demonstrated that several biotic factors can positively influence biofilm formation of the *A. brasilense* strain used. One bacterium used, namely *P. putida* X236 has the capability of influencing directly, possibly via secondary metabolites. This strain also showed to be a good combination for a potential future co-inoculum along with *A. brasilense.* Nevertheless, further research is required to verify which compounds produced by *P. putida* X236 influenced *A. brasilense* biofilm formation and further testing ensure positive effects exists on selected plants. Root exudates from mycorrhized plants did not influenced biofilm formation, though two mycorrhizal fungal species, *G. mosseae* and *G. claroideum*, proved to have a role in *A. brasilense* chemotaxis. IAA synthesis, which is one of the major plant benefits attributed to *A. brasilense*, and further research is required either in the pathways specifics as in tryptophan independent pathway or synthesis in biofilm and its differences to planktonic cells.

The main goal was achieved, and interspecific interactions were inquired. This work highlights the potential for using a microbial inoculum consortium consisting of mycorrhizal fungi and bacterial species to stimulate biofilm development and function of *A. brasilense.*

VI - References

1- Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nature Review of Microbiology. 11: 789-799

2- Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JG, Dasgupta M, Marrie TJ. (1987). Bacterial biofilms in nature and disease. Annual Review of Microbiology. 41: 435-464

3- O'Toole G, Kaplan HB, Kolter R. (2000). Biofilm Formation as Microbial Development. Annual Review of Microbiology. 54: 49-79

4- Solano C, Echeverz M, Lasa I. (2014). Cell regulation biofilm dispersion and quorum sensing. Current Opinion in Microbiology. 18: 96-104

5- Miller MB, Bassler BL. (2001). Quorum sensing in bacteria. Annual Review of Microbiology. 55: 165-199

6- Kaplan HB, Greenberg EP. (1985). Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. Journal of Bacteriology. 163: 1210-1214

7- Christensen BE. (1989). The role of extracellular poly-saccharides in biofilms. Journal of Biotechnology. 10: 181-202

8- Gilbert P, Das J, Foley I. (1997). Biofilms susceptibility to antimicrobials. Advances in Dental Research. 11: 160–167

9- Nichols WW, Dorrington SM, Slack MPE, Walmsley HL. (1988). Inhibition of tobramycin diffusion by binding to alginate. Antimicrobial Agents and Chemotherapy. 32: 518-523

10- Nichols WW, Evans MJ, Slack MPE, Walmsley HL. (1989). The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa.* Journal of General Microbiology. 135: 1291-1303

11- Decho AW. (1990). Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanography and Marine Biology - an Annual Review. 28: 73-153

12- Flemming HC. (1993). Biofilms and environmental protection. Water Science and Technology. 27: 1-10

13- Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. (1995). Microbial biofilms. Annual Review of Microbiology. 49: 711-745

14- Ehlers LJ, Bouwer EJ. (1999). RP4 plasmid transfer among species of *Pseudomonas* in a biofilm reactor. Water Science and Technology. 7: 163-171.

15- Hausner M, Wuertz S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Applied and Environmental Microbiology. 65: 3710-3713

16- Danhorn T, Fuqua C. (2007). Biofilm formation by plant-associated bacteria. Annual Review of Microbiology. 61: 401-422

17- Kloepper JW, Schroth MN. (1978). Plant growth-promoting rhizobacteria on radishes. Proceedings of the 4th international conference on plant pathogenic bacteria (Angers, France: Station de Pathologie Végétale et Phytobactériologie). Institut National de la Recherche Agronomique. 2: 879–882

18- Kloepper JW, Scher FM, Laliberte B, Tipping B. (1986). Emergence-plant growth promoting rhizobacteria: description and implication for agriculture. Iron, siderophores, and plant diseases. Swinburne TR eds. Springer Life Science, The Netherlands

19- Davison J. (1988). Plant beneficial bacteria. Bio-Technology. 6: 182-286

20- Okon Y, Labandera-Gonzalez CA. (1994). Agronomic applications of *Azospirillum*: an evaluation of 20 years of worldwide field inoculation. Soil Biology & Biochemistry. 26: 1591–1601

21- Bashan Y, Levanony H. (1990). Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Canadian Journal of Microbiology. 36: 591–608

22- Bashan Y, Ream Y, Levanony H, Sade A. (1995). Non-specific responses in plant growth, yield and root colonization of noncereal crop plants to inoculation with *Azospirillum brasilense* Cd. Canadian Journal of Botany. 67: 1317–1324

23- Baca BE, Elmerich C. (2007). Microbial production of plants hormones by microorganisms. Associative nitrogen-fixation bacteria and cyanobacteria. IV. Series: fitrogen fixation: origins, Applications, and Research Progress. 113–137. Elmerich C, Newton W, eds. Springer Life Science, The Netherlands

24- Steenhoudt O, Vanderleyden J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. Federation of European Microbiological Societies Microbiology Reviews. 24: 487-506

25- Creus CM, Graziano M, Casanovas EM, Pereyra MA, Simontacchi M, Puntarulo S, Barassi CA, Lamattina L. (2005). Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. Planta. 221: 297-303

26- Bakanchikova TI, Lobanok EV, Pavlova-Ivanova LK, Redkina TV, Nagapetyan ZA, Majsuryan AN. (1993). Inhibition of tumor formation process in dicotyledonous plants by *Azospirillum brasilense* strains. Mikrobiologiya. 62: 515–523

27- Romero AM, Correa O, Moccia S, Rivas JG. (2003). Effect of *Azospirillum*-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato. Journal of Applied Microbiology. 95: 832–838

28- Bashan Y, de-Bashan LE. (2002). Protection of tomato seedlings against infection by *Pseudomonas syringae* pv. Tomato by using the plant growth-promoting bacterium *Azospirillum brasilense.* Applied and Environmental Microbiology. 68: 2637–2643

29- Bashan Y, de-Bashan LE. (2002). Reduction of bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato by combined treatments of plant growth-promoting bacterium, *Azospirillum brasilense*, streptomycin sulfate, and chemo-thermal seed treatment. European Journal Plant Pathology. 108: 821–829

30- Somers E, Ptacek D, Gysegom P, Srinivasan M, Vanderleyden J. (2005). *Azospirillum brasilense* produces the auxin-like phenylacetic acid by using the key enzyme for indole-3-acetic acid biosynthesis. Applied and Environmental Microbiology. 71: 1803-10

31- Hartmann A, Fu HA, Burris RH. (1988). Influence of amino acids on nitrogen fixation ability and growth of *Azospirillum* spp. Applied and Environmental Microbiology. 54: 87-93

32- Reinhold B, Hurek T, and Fendrik I, (1985). Strain specific chemotaxis of *Azospirillum* spp. Journal of Bacteriology. 162: 190-195

33- Vial L, Cuny C, Gluchoff-Fiasson K, Comte G, Oger PM, Faure D, Dessaux Y, Bally R, Wisniewski-Dyé F. (2006). N-acyl-homoserine lactone-mediated quorum-sensing in *Azospirillum*: an exception rather than a rule. Federation of European Microbiological Societies Microbiology Ecology. 58: 155–168

34- Somers E, Ptacek D, Gysegom P, Srinivasan M, Vanderleyden J. (2005). *Azospirillum brasilense* produces the auxin-like phenylacetic acid by using the key enzyme for indole-3-acetic acid biosynthesis. Applied and Environmental Microbiology. 71: 1803-1810

35- Greer-Phillips SE, Stephens BB, Alexandre G. (2004). An energy taxis transducer promotes root colonization by *Azospirillum brasilense*. Journal of Bacteriology. 186: 6595-6604

36- Burdman S, Okon Y, Jurkevitch E. (2000). Surface characteristics of *Azospirillum brasilense* in relation to cell aggregation and attachment to plant roots. Critical Reviews in Microbiology. 26: 91-110

37- Burdman S, Jurkevitch E, Schwartsburd B, Okon Y. (1999). Involvement of outermembrane proteins in the aggregation of *Azospirillum brasilense*. Microbiology. 145:1145–52

38- Burdman S, Dulguerova G, Okon Y, Jurkevitch E. (2001). Purification of the major outer membrane protein of *Azospirillum brasilense*, its affinity to plant roots, and its involvement in cell aggregation. Molecular Plant-Microbe Interactions. 14: 555-561

39- Wisniewski-Dyé F, Borziak K, Khalsa-Moyers G, Alexandre G, Sukharnikov LO, Wuichet K, Hurst GB, McDonald WH, Robertson JS, Barbe V, Calteau A, Rouy Z, Mangenot S, Prigent-Combaret C, Normand P, Boyer M, Siguier P, Dessaux Y, Elmerich C, Condemine G, Krishnen G, Kennedy I, Paterson AH, González V, Mavingui P, Zhulin IB. (2011). *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. Public Library of Science Genetics. 7(12): e1002430. doi: 10.1371/journal.pgen.1002430

40- Combes-Meynet E, Pothier JF, Moënne-Loccoz Y, Prigent-Combaret C. (2011) The *Pseudomonas* secondary metabolite 2, 4-diacetylphloroglucinol is a signal inducing rhizoplane expression of *Azospirillum* genes involved in plant-growth promotion. Molecular Plant-Microbe Interactions. 24: 271-284

41- Di Palma AA, Pereyra CM, Ramirez LM, Xiqui Vazquez ML, Baca BE, Pereyra MA, Lamattina L , Creus CM. (2013). Denitrification-derived nitric oxide modulates biofilm formation in *Azospirillum brasilense*. Federation of European Microbiological Societies Microbiology Letters. 338: 77-85

42- Smith SE, Read DJ. (2008). Mycorrhizal Symbiosis 3rd eds. Academic Press, Amsterdam
43- Schüssler A, Schwarzott D, Walker C. (2001). A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycological Research. 105:1413–1421
44- Tedersoo L, May TW, Smith ME. (2010). Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza. 20: 217-263
45- Fitter AH, Garbays J. (1994). Interactions between mycorrhizal fungi and other soil organisms. Plant and Soil. 159: 123-132
46- Mishra S, Sharma S, Vasudevan P. (2008). Comparative effect of biofertilizers on fodder production and quality in guineagrass (*Panicum maximum* Jacq.). Journal of the Science of Food and Agriculture. 88: 1667-1673
47- Hayman DS. (1982). Influence of soils and fertility on activity and survival of vesicular-arbuscular mycorrhizal fungi. Phytopathology. 72: 1119–1125
48- Andrade G., Mihara KL, Linderman RG, Bethlenfalvay, GJ. (1997). Bacteria from rhizosphere and hyphosphere soils of different arbuscular-mycorrhizal fungi. Plant and Soil. 192: 71-79
49- Artursson V, Finlay RD, Jansson JK. (2005) Combined bromodeoxyuridine immunocapture and terminal restriction fragment length polymorphism analysis highlights differences in the active soil bacterial metagenome due to *Glomus mosseae* inoculation or plant species. Environmental Microbiology. 7: 1952–1966
50- Drigo B, Pijl AS, Duyts H, Kielak AM, Gamper HA, Houtekamer MJ, Boschker HTS, Bodelier PLE, Whiteley AS, van Veen JA, Kowalchuk GA. (2010). Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO_2. Proceedings of the National Academy of Sciences. 107: 10938-10942
51- Validov S, Kamilova F, Qi S, Stephan D, Wang JJ, Makarova N, Lugtenberg B. (2007). Selection of bacteria able to control *Fusarium oxysporum* f. Sp. *Radicis lycopersici* in stonewool substrate. Journal of Applied Microbiology. 102: 461-471
52- de Boer M, Bom P, Kindt F, Keurentjes JJ, van der Sluis l, van Loon LC, Bakker PA. (2003). Control of *Fusarium* Wilt of Radish by Combining *Pseudomonas putida* Strains that have Different Disease-Suppressive Mechanisms. Phytopathology. 93: 626-632
53- Weller DM, Thomashow LS. (1993). Microbial metabolites with biological activity against plant pathogens, Pest Management: Biologically Based Technologies. 173-180. Lumsden RD, Vaughn JL eds. American Chemical Society, Washington DC
54- Kamilova F, Validov S, Azarova T, Mulders I, Lugtenberg B. (2005). Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environmental Microbiology. 7: 1809–1817
55- Cavaglieri L, Orlando J, Rodríguez MI, Chulze S, Etcheverry M. (2005).Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level. Research in Microbiology. 156: 748-54
56- Kildea S, Ransbotyn V, Khan MR,Fagan B, Leonard G, Mullins E. (2008). *Bacillus megaterium* shows potential for the biocontrol of *septoria tritici* blotch of wheat. Biological Control. 47: 37-45
57- Lee JP, Lee SW, Kim CS, Son JH, Song JH, Lee KY, Kim HJ, Jung SJ, Moon BJ. (2006). Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea.* Biological Control. 37: 329-337
58- Huang X, Zhang N, Yong X, Yang X, Shen Q .(2012). Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiological Research. 167: 135-43
59- Xie ZP, Staehelin C, Vierheilig H, Wiemken A, Jabbouri S, Broughton WJ, Vogeli-Lange R, T Boller. (1995). Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiology. 108: 1519-1525
60- Cassána F, Perriga D, Sgroya V, Pennab O, Lunaa V. (2009). *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109 inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). European Journal of Soil Biology. 45: 28-35
61- Paterson E, Gebbing T, Abel C, Sim A, Telfer G. (2007). Rhizodeposition shapes rhizosphere microbial community structure in organic soil. New Phytologist. 173: 600-610
62- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. (2006). The role of root exudates in rizosphere interactions with plants and other organisms. Annual Review of Plant Biology. 57: 233-66

63- Bakker PA, Berendsen RL, Doornbos RF, Wintermans PC, Pieterse CM. (2013). The rhizospere revisited: root microbiomics. Frontiers in Plant Science. 4: 165

64- Haichar F, Marol C, Berge O, Rangel-Castro J, Prosser J. (2008). Balesdent J, Heulin T, Achouak W. Plant host habitat and root exudates shape soil bacterial community structure. International Society for Microbial Ecology. 2: 1221-1230

65- Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA. (2014). Induced systemic resistance by beneficial microbes. Annual Review of Phytopathology. 52: 347-375

66- Trabelsi D, Mhamdi R. (2013). Microbial inoculants and their impact on soil microbial communities: A Review Hindawi BioMed Research International. Vol. 2013 Article ID 863240

67- Morrissey JP, Dow JM, Mark GL, O'Gara F. (2004). Are microbes at the root of a solution to world food production? European Molecular Biology Organization reports. 5: 922-926

68- Reis VM, dos Santos Teixeira KR, Pedraza RO. (2011). Bacteria in agrobiology: plant growth responses. 6: 132-134. Maheshwari DK eds. Springer Life Science, The Netherlands

69- Lerner A, Herschkovitz Y, Baudoin E. (2006). Effect of *Azospirillum brasilense* inoculation on rhizobacterial communities analyzed by denaturing gradient gel electrophoresis and automated ribosomal intergenic spacer analysis. Soil Biology and Biochemistry. 38: 1212-1218

70- de Salamone I, Di Salvo LP, Ortega J, Boa Sorte P, Urquiaga S, Teixeira KR. (2010). Field response of rice paddy crop to *Azospirillum* inoculation: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different parts of the plants. Plant and Soil. 336: 351-362

71- Felici C, Vettori L, Giraldi E, Forino LM, Toffanin A, Tagliasacchi AM, Nuti M. (2008). Single and co-inoculation of *Bacillus subtilis* and *Azospirillum brasilense* on *Lycopersicon esculentum*: effects on plant growth and rhizosphere microbial community. Applied Soil Ecology. 40: 260-270

72- Kozdrój J, Trevors JT, Van Elsas JD. (2004). Influence of introduced potential biocontrol agents on maize seedling growth and bacterial community structure in the rhizosphere. Soil Biology and Biochemistry. 36: 1775–1784

73- Ruíz-Sánchez M, Armada E, Munoz Y, de Salamone I, Aroca R, Ruíz-Lozano J, Azcón R. (2011). *Azospirillum* and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions. Journal of Plant Physiology. 168: 1031-1037

74- Pulido LE, Cabrera A, Medina N. (2003). Biofertilization using rhizobacteria and AMF in the production of tomato (*Lycopersicon esculentum* Mill.) and onion (*Allium cepa* L.) seedlings. II. Root colonization and nutritional status. Cultivos Tropicales. 24: 5-13

75- Lapage S, Shelton J, Mitchell T. (1970). Methods in Microbiology. Norris J. and Ribbons D eds. Academic Press, London.

76- Allen EK, Allen ON. (1950). Biochemical and symbiotic properties of the rhizobia. Bacteriological Reviews. 14: 273-330

77- O'Toole GA. (2011). Microtiter dish biofilm formation assay. Journal of Visualized Experiments. 47: 2437

78- Niu C, Gilbert ES. (2004). Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Applied and Environmental Microbiology. 70: 6951-6956

79- Donati AJ, Lee HI, Leveau JH, Chang WS. (2013). Effects of indole-3-acetic acid on the transcriptional activities and stress tolerance of *Bradyrhizobium japonicum*. Public Library of Science ONE. 8(10): e76559. doi:10.1371/journal.pone.0076559

80- Hoagland DR, Arnon DI. (1950). The water-culture method of growing plants without soil. California Agricultural Experiment Station. 347.

81- Pinior A, Wys U, Piche ÂY, Vierheilig H. (1999). Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Canadian Journal of Botany 77: 1-7.

82- de Weert S, Kuiper I, Lagendijk EL, Lamers GE, Lugtenberg BJ. (2004). Role of chemotaxis toward fusaric acid in colonization of hyphae of *Fusarium oxysporum* f. sp. *radicis-lycopersici* by *Pseudomonas fluorescens* WCS365. Molecular Plant-Microbe Interactions. 16: 1185-1191

83- Gordon SA, Weber RP. (1951). Colorimetric estimation of indolacetic acid. Plant Physiology. 26: 192-195

84- Sarwar M, Kremer RJ. (1995). Determination of bacterially derived auxins using a microplate method. Letters in Applied Microbiology. 20: 282-285

85- Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada

M, Tabata S. (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Research. 9: 189-197

86- Yates MG, de Souza EM, Kahindi JH. (1997). Oxygen, hydrogen and nitrogen fixation in *Azotobacter.* Soil Biology and Biochemistry. 29: 863-869

87- Shimoda Y, Shinpo S, Kohara M, Nakamura Y, Tabata S, Sato S. (2008). A large scale analysis of protein-protein interactions in the nitrogen-fixing bacterium *Mesorhizobium loti.* DNA Research. 15: 13-23

88- Walker V, Bertrand C, Bellvert F, Moënne-Loccoz Y, Bally R, Comte G. (2011). Host plant secondary metabolite profiling shows a complex, strain-dependent response of maize to plant growth-promoting rhizobacteria of the genus *Azospirillum.* New Phytologist. 189: 494-506

89- Song YY, Cao M, Xie LJ, Liang XT, Zeng RS, Su YJ, Huang JH, Wang RL, Luo SM. (2011). Induction of DIMBOA accumulation and systemic defense responses as a mechanism of enhanced resistance of mycorrhizal corn (*Zea mays* L.) to sheath blight. Mycorrhiza. 21: 721-731

90- Neal AL, Ahmad S, Gordon-Weeks R, Ton J. (2012). Benzoxazinoids in Root Exudates of Maize Attract *Pseudomonas putida* to the Rhizosphere. Public Library of Science ONE. 7(4): e35498. doi: 10.1371/journal.pone.0035498

91- Chen KJ, Zheng YQ, Kong CH, Zhang SZ, Li J, Liu XG. (2010). 2, 4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) and 6-Methoxy-benzoxazolin-2-one (MBOA) levels in the wheat rhizosphere and their effect on the soil microbial community structure. Journal of Agricultural and Food Chemistry. 58: 12710-12716

92- Macias FA, Oliveros-Bastidas A, Marin D, Castellano D, Simonet AM, Molinillo JM. (2004). Degradation studies on benzoxazinoids. Soil degradation dynamics of 2, 4-dihydroxy-7-methoxy-(2H)-1, 4-benzoxazin-3(4H)-one (DIMBOA) and its degradation products, phytotoxic allelochemicals from Gramineae. Journal of Agricultural and Food Chemistry. 52: 6402-6413

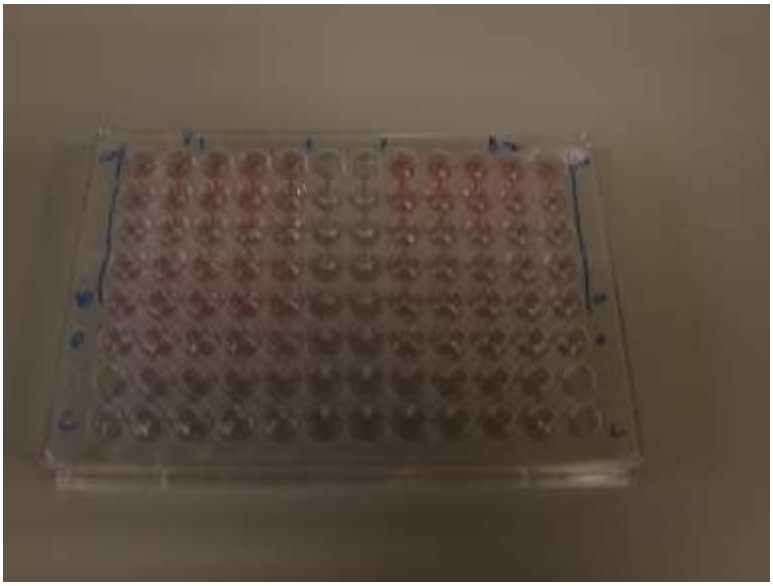
93- Zhulin IB, Tretyakova SE, Ionatov VV. (1988). Chemotaxis of *Azospirillum brasilense* towards compounds typical of plant root exudates. Folia Microbiologica. 33: 277-28

94- Spaepen S, Vanderleyden J, R Remans. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. Federation of European Microbiological Societies Microbiology Reviews. 31: 425-448

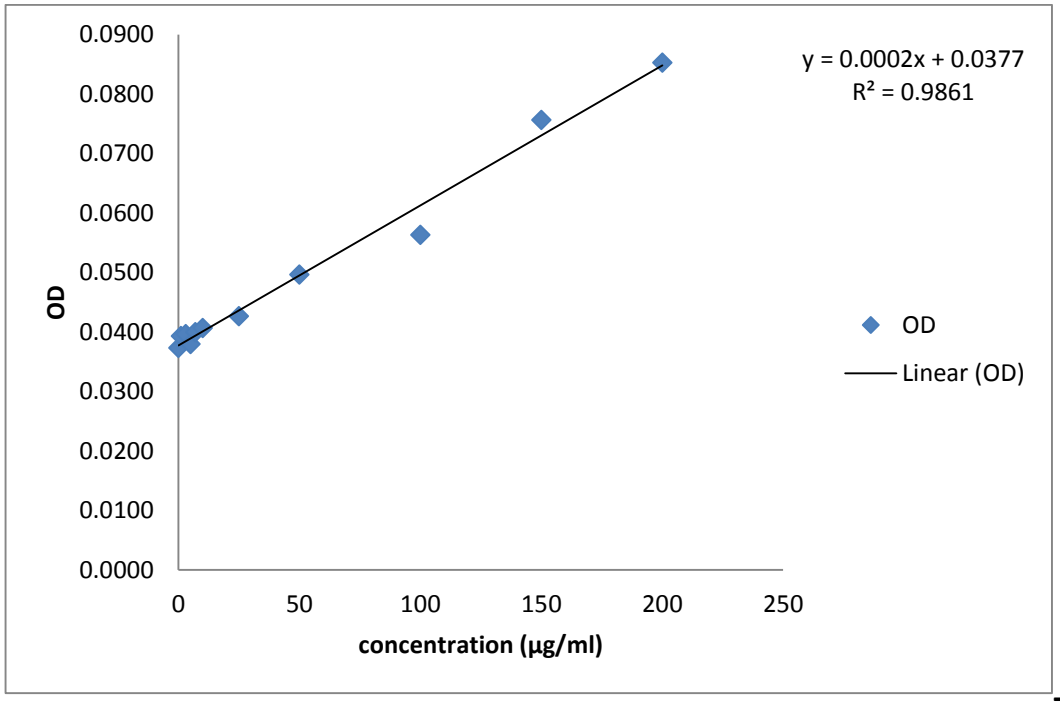
95- Jaeger JH, Lindow SE, Miller S, Clark E, Firestone MK. (1999). Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Applied and Environmental Microbiology. 65: 2685–2690

VII - Appendices

Appendix I: Sterile system used in root exudate assay. Plants were inoculated with *B. japonicum*, *P. putida* X236 or *A. brasilense*.



Appendix II: Microtiter plate of Indol-3-acetic-acid quantification assay, the pink color was caused by reaction with Salkowski reagent. Afterwards optic density was measured at $OD_{530.}$



Appendix III: Calibration curve of IAA. Optic density of several solutions with known concentration of IAA were used to yield the equation y = 0.0002x + 0.0377. With the equation and the values of optic density obtained, IAA concentration of the samples was calculated. Subtitles: OD, optic density; X-axis, concentration of IAA.